

U.S. PATENT APPLICATION

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Invention: SUBSTRATES OF N-END RULE UBIQUITYLATION AND METHODS
FOR MEASURING THE UBIQUITYLATION OF THESE SUBSTRATES

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SPECIFICATION

5 **SUBSTRATES OF N-END RULE UBIQUITYLATION AND METHODS FOR**
 MEASURING THE UBIQUITYLATION OF THESE SUBSTRATES

1. **CROSS REFERENCES TO RELATED APPLICATIONS**

 This application claims priority to U.S. Provisional Application No. 60/422,448,
10 filed October 30, 2002 and U.S. Provisional Application No. 60/486,529 filed July 19,
2003, which are incorporated hereby by reference.

2. **FIELD OF INVENTION**

 The present invention relates to detecting and measuring protein ubiquitylation
15 via the N-end rule pathway and to identifying novel targets, enzymes and modulators of
N-end rule ubiquitylation.

3. **BACKGROUND**

 The ability to degrade proteins is an essential function of all eukaryotic cells. The
20 ubiquitin-proteasome system has evolved to play an active role in cellular quality control
by selective degradation of normal or damaged proteins. The ubiquitin-proteasome
system is fundamental to cell cycle control, transcriptional regulation, stress response,
immune and inflammatory responses and other vital processes (Hershko and
Ciechanover, 1998, *Annu. Rev. Biochem.*, 67:425-479; Varshavsky, 1997, *Trends*
25 *Biochem. Sci.*, 22: 383-387; Hochstrasser, 1996, *Annu. Rev. Genet.*, 30: 405-439).

 Ubiquitin (Ub) is a highly conserved 76-amino acid protein. Protein degradation
via the ubiquitin-proteasome pathway generally involves covalent attachment of multiple

5 molecules of ubiquitin to the protein substrate. The protein substrate is subsequently degraded by the 26S proteasome complex, and the free ubiquitin is recycled. There are also examples of proteins whose functions appear to be regulated by ubiquitylation, although ubiquitylation does not appear to target them for degradation (Hwang et al., 2003, *Mol. Cell*, 11: 261-266).

10 The attachment of ubiquitin to many known substrate proteins is believed to occur in a series of enzymatic reactions carried out sequentially by three classes of proteins: (1) an ubiquitin-activating enzyme (E1) activates ubiquitin in an ATP-dependent manner to form a thioester bond between the carboxy-terminal Gly of Ub and a Cys residue of E1; (2) activated Ub is then transferred to an ubiquitin-conjugating enzyme (E2 or UBC) to
15 form another thioester bond; (3) a ubiquitin ligase (E3) catalyzes or promotes, in a substrate specific manner, the transfer of Ub from the E2 to a Lys residue of the substrate protein to form an isopeptide bond. An internal Lys residue of Ub can also form an isopeptide bond with the C-terminus of another Ub to create a multi-Ub chain that serves as a targeting signal for proteasome.

20 In addition to ubiquitylation, proteins can be modified by attachment of ubiquitin-like proteins (such as Sentrin/SUMO or NEDD8) through distinct pathways that may have physiological roles distinct from the ubiquitylation pathway (Yeh et al., 2000, *Gene*, 248: 1-14). While there are at least 25 mammalian E2 family members, some poorly characterized, the number of different E3 enzymes is predicted to gross over a hundred
25 (Weissman, 2001, *Nature Reviews*, 2: 169-178). E3 ubiquitin ligases come in a variety of different structural classes (such as HECT and RING finger) and act via a number of distinct pathways. So far, most E3 proteins that have been shown to interact with E2s

5 and to mediate ubiquitylation in *in vitro* systems lack defined substrates other than themselves. The currently available information on E3 identification and specificity is insufficient to develop clear understanding of the role of many E3s in biological processes and disease.

The defective regulation of the ubiquitin-proteasome system manifests in diseases
10 that range from developmental abnormalities and autoimmunity to neurodegenerative diseases and cancer (Weissman, 2001, *Nature Reviews*, 2: 169-178). The discovery of HECT E3s was a direct consequence of the finding that oncogenic strains of human papillomavirus (HPV) encode isoforms of a protein called E6, which specifically inactivates the tumor suppressor protein p53. E6 serves as an adaptor between p53 and
15 an E6-associated E3 that catalyzes the ubiquitylation of p53 (Scheffner et al., 1993, *Cell*, 75:495-505). Mutations in the same HECT E3 enzyme are shown to give rise to Angelman syndrome, a severe neurologic disorder (Kishino et al., 1997, *Nature Genet.*, 15: 70-73). Prominent among RING E3s is a product of breast and ovarian cancer susceptibility gene (BRCA1). Mutations in this protein are found in familial forms of
20 breast and ovarian cancer (Brzovic et al., 1998, *J. Biol. Chem.*, 273: 7795-7799). Among well-studied RING E3s are the oncoprotein MDM2, an E3 ligase that ubiquitylates p53 and upon overexpression may lead to cancer; the proto-oncoprotein c-Cbl which ubiquitylates growth factor receptors (Waterman et al., 1999, *J. Biol. Chem.*, 274: 22151-22154; Joazeiro et al., 1999, *Science*, 286: 309-312; Yokouchi et al., 1999, *J. Biol. Chem.*,
25 274: 31707-31712) and the inhibitors of apoptosis (IAP) proteins (Yang et al., 2000, *Science*, 288: 874-877; Hwang et al., 2000, *J. Biol. Chem.*, 275: 26661-26664). Mutations in Parkin, another RING finger E3, are associated with juvenile Parkinson's

5 disease (Shimura et al., 2000, *Nature Genet.*, 25: 302-305; Chung et al., 2001, *Nature Med.*, 7(10): 1144-1150).

One specific example of an important ubiquitylation pathway is N-End rule ubiquitylation, and especially N-End rule ubiquitylation where ubiquitylation is preceded by N-terminal segment cleavage, where the N-terminal segment comprises one or more
10 amino acid residues. The proteolysis exposes an N-degron which comprises a destabilizing N-terminal residue plus an internal Lys residue where a multi-Ub chain is later attached. The N-terminal segment is cleaved to form an activated substrate of the Ub-dependent N-end rule pathway (activated fragment) which is recognized through exposed destabilizing N-terminal residue.

15 The N-end rule pathway has been the subject of several review articles; see, e.g., Varshavsky, 1996, *Proc. Natl. Acad. Sci. U.S.A.*, 93: 12142. The ubiquitin ligase UBR1, an E3 ligase, has been shown to ubiquitylate N-end rule substrates and has two binding sites for primary destabilizing N-terminal residues. The type I site is specific for basic N-terminal residues Arg, Lys, His. The type II site is specific for bulky hydrophobic
20 residues Phe, Leu, Trp, Tyr, and Ile. Dipeptides carrying type I or type II N-terminal residues can serve as inhibitors of ubiquitylation of the corresponding type I or type II N-end rule substrates (Gonda et al., 1989, *J. Biol. Chem.*, 264: 16700-16712). UBR1 from yeast contains yet another substrate-binding site, which recognizes proteins for ubiquitylation through an internal recognition site on substrates; this process can be
25 enhanced by the presence of type I and type II dipeptides (Turner et al., 2000, *Nature*, 405: 579-583).

5 The degradation signal for ubiquitylation via the N-end rule pathways is termed
an N-degron and comprises the primary destabilizing N-terminal residue and an internal
lysine which is the site of ubiquitylation. Destabilizing N-terminal residues can be
generated through proteolytic cleavages of specific proteins and other N-terminal
modifications which reveal destabilizing residues at the new N-terminus. The residues
10 that are exposed or modified to reveal an N-degron have been termed a pre-N-degron or
pro-N-degron. For example, Sindbis virus RNA polymerase is produced during viral
infection through site-specific cleavage of the viral polyprotein precursor and carries an
N-terminal Tyr that has been shown in rabbit reticulocyte lysates to target the protein for
ubiquitylation via the N-end rule pathway (deGroot et al., 1991, *Proc. Natl. Acad. Sci.*
15 *U.S.A.*, 88: 8967-8971). Another example is RGS4, whose N-terminal degradation signal
is generated through a series of N-terminal modifications including (i) removal of N-
terminal Met and exposure of Cys-2 at the N-terminus, (ii) oxidation of Cys-2 into
cysteic acid, and (iii) conjugation of Arg to the N-terminus of the protein (Kwon et al.,
2002, *Science*, 297: 96-99).

20 Very few N-end rule substrates are characterized to date. However, recently
discovered N-end rule substrates linked to disease or pathology demonstrate the
biological importance of N-end rule ubiquitylation pathway. For example, a carboxy-
terminal fragment of cohesin in *Saccharomyces cerevisiae* is a physiological substrate for
the ubiquitin/proteasome-dependent N-end rule pathway. Overexpression of this
25 fragment is lethal and, in cells that lack an N-end rule ubiquitylation pathway, a highly
increased frequency of chromosome loss is detected (Rao et al., 2001, *Nature*, 410: 955-
959). Recent studies also indicate that enhanced protein breakdown in skeletal muscle

5 leading to muscle wasting in patients with acute diabetes results from an accelerated Ub conjugation and protein degradation via the N-end rule pathway. The same pathway is activated in cancer cachexia, sepsis and hyperthyroidism (Lecher et al., 1999, *J. Clin. Invest.*, 104: 1411-1420; Solomon et al., 1998, *Proc. Natl. Acad. Sci. USA*, 95: 12602-12607).

10 Aprataxin is a member of the HIT (histidine triad) protein family, named for the H ϕ H ϕ H ϕ motif, where ϕ is a hydrophobic amino acid (Brenner, 2002, *Biochemistry*, 41(29): 9003-9014). HIT is a superfamily of nucleotide hydrolases and transferases, which act on the alpha-phosphate of ribonucleotides, and contain an approximately 30 kDa domain that is typically a homodimer of approximately 15 kDa polypeptides with
15 two active sites. The superfamily is also generally said to include GalT-like proteins even though these contain a slightly different motif (HXHXQ $\phi\phi$), the motif being repeated twice in a single polypeptide chain that retains a single active site.

Members of the HIT superfamily of proteins have representatives in all cellular life. On the basis of sequence, substrate specificity, structure, evolution, and mechanism,
20 HIT proteins can be classified into the Hint branch, which consists of adenosine 5'-monophosphoramidate hydrolases, the Fhit branch, which consists of diadenosine polyphosphate hydrolases, and the GalT branch, which consists of specific nucleoside monophosphate transferases, including galactose-1-phosphate uridylyltransferase, diadenosine tetraphosphate phosphorylase, and adenylyl sulfate:phosphate
25 adenylyltransferase. A loss of at least one human representative of each branch is associated with a human disease (Brenner, 2002, *Biochemistry*, 41: 9003-9014). Fhit is lost early in the development of many epithelially derived tumors. GalT is deficient in

5 galactosemia. Aprataxin, a Hint branch hydrolase, is mutated in ataxia-oculomotor
apraxia syndrome (Date et al., 2001, *Nature Genetics*, 29: 184-188; Moreira et al., 2001,
Nature Genetics, 29: 189-193), which is the most common autosomal recessive
neurodegenerative disease among Europeans and people of European descent and the
most frequent cause of autosomal recessive ataxia in Japan. Recent studies in patients
10 with early-onset ataxia identified one insertion and two missense mutations in the
aprataxin gene product (Shimazaki et al., 2002, *Neurology*, 59: 590-595). It has been
suggested that aprataxin is involved in DNA repair and therefore its regulation is crucial
for cancer predisposition and cerebellar neuron survival (Moreira et al., 2001, *Nature
Genetics*, 29: 189-193; Durocher et al., 2000, *Mol. Cell*, 6: 1169-1182). The molecular
15 targets and pathways involving aprataxin remain to be discovered. With identification of
such targets and pathways, it is hoped that new light can be shed on brain development
and motor coordination.

Microtubule-associated protein tau (MAPT or tau) is a protein that is believed to
play a role in a variety of disease processes. The gene encoding tau undergoes complex,
20 regulated alternative splicing, which gives rise to several mRNA species. Six tau
isoforms are produced in adult human brain by alternative mRNA splicing from a single
gene. The isoforms differ from each other by the presence or absence of 29-amino acid
or 58-amino acid inserts located in the N-terminal half and a 31-amino repeat located in
the C-terminal half. Inclusion of the latter, which is encoded by exon 10 of the tau gene,
25 gives rise to three tau isoforms with four repeats each; the other three isoforms have three
repeats each (Kosik et al., 1989, *Neuron*, 2: 1389-1397; Goedert et al., 1989, *Neuron*, 3:
519-526). The repeats and some adjoining sequences constitute the microtubule-binding

5 domains of tau. Similar levels of 3-repeat and 4-repeat tau isoforms are found in normal cerebral cortex. The tau filaments from Alzheimer disease brain contain all six tau isoforms in a hyperphosphorylated state. At the same time, it is shown that the ratio of 3-repeat to 4-repeat tau isoforms is an important determinant of the ratio of microtubule-bound and free forms of tau (Lu et al., 2001, *Mol. Biol. Cell*, 12: 171-184). It has also
10 been demonstrated that longer 4-repeat tau isoforms have approximately 4.5 times higher affinity to microtubules and 2-3 fold faster rate of microtubule assembly than 3-repeat tau isoforms (Goedart and Jakes, 1990, *EMBO J*, 9(13): 4225-4230; Butner and Kirschner, 1991, *J. Cell Biol.*, 115(3): 717-730; Gustke et al., 1992, *FEBS Lett.*, 307(2): 199-205).

MAPT transcripts (including splice isoforms and mutations) are differentially
15 expressed in the nervous system, depending on the stage of neuronal maturation and neuron type. The shortest of the six tau isoforms termed 3R0N is specifically abundant in a fetal brain (Goedert et al., 1989, *Neuron*, 3(4): 519-526). In fact, the human brain expresses only 3R0N isoforms that are highly phosphorylated until the postnatal period, and this may imply a specific role of this isoform during axonal growth and
20 synaptogenesis (Kosik et al., 1989, *Neuron*, 2(4): 1389-1397).

Mutations in tau result in several neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Pick's disease, frontotemporal dementia, cortico-basal degeneration and progressive supranuclear palsy as well as subcortical gliosis and pallido-nigro-luydian degeneration (Lu et al., 2001, *Mol. Biol. of Cell*, 12: 171-184;
25 Golbe and Lazzarini, 2001, *Mov. Disord.*, 16(3): 442-7 and references therein).

Neurofibrillary tangles made predominantly from intracellular bundles of self-assembled hyperphosphorylated tau proteins are the hallmark features of Alzheimer's

5 disease (Mandelkow et al., 1995, *Neurobiol Aging*, 16(3): 347-354). Studies on the
microtubule-associated protein tau in Alzheimer disease have noted that, in the brains of
patients with Alzheimer disease, the neuronal cytoskeleton is progressively disrupted and
replaced by neurofibrillary tangles of paired helical filaments (PHFs), composed mainly
of hyperphosphorylated forms of tau (also called 'AD P-tau'). It has also been
10 demonstrated that in solution normal tau associates with the hyperphosphorylated AD P-
tau to form large tangles of filaments and that dephosphorylation with alkaline
phosphatase abolished the ability of AD P-tau to aggregate *in vitro* (Johnson and Bailey,
2002, *J. Alzheimer Disease*, 4: 375-398).

It has also been shown that elevated levels of tau inhibit intracellular transport in
15 neurons, particularly the plus-end-directed transport by kinesin motors from the center of
the cell body to the neuronal processes (Ebner et al., 1998, *J. Cell Biol.*, 143(3): 777-
794). This inhibition is significant because critical organelles, such as peroxisomes,
mitochondria, and transport vesicles carrying supplies for the growth cone, are unable to
penetrate the neurites, leading to stunted growth, increased susceptibility to oxidative
20 stress, and likely pathologic aggregation of proteins such as amyloid precursor protein. It
has been concluded that the tau:tubulin ratio is normally low, and that increased levels of
tau become detrimental to the cell (Ebner et al., 1998, *J. Cell Biol.*, 143(3): 777-794).

Synaptotagmin-like proteins (SLPs) are a subfamily of the C2 domain-containing
family of proteins and have a high degree of homology to synaptotagmin. The proteins
25 contain two conserved domains at the N-terminus (referred to as SLP homology domains
1 and 2 or SHD1 and 2) and two carboxyl-terminal Ca^{2+} -binding motifs (C2 domains)
(Pallanck, 2003, *TRENDS Neurosci.*, 26(1): 2-4). The SHD has also been found in other

5 proteins including SLP homologs lacking C2 domains (Slac2). Found in phospholipases and protein kinase C, C2 domains have also been identified in synaptotagmins, a family of proteins involved in regulating neurotransmission. The function of synaptotagmin, as a calcium sensor in SNARE-mediated exocytosis, is extremely complex and finely regulated to allow for coupling the calcium signal to the fast synaptic vesicle exocytosis, which leads to speculations that synaptotagmin evolved to acquire a function beyond calcium/phospholipids binding (Rickman and Davletov, *J. Biol. Chem.*, 2002, [epub]; Yoshihara et al., 2002, *Neuron*, 36: 897-908; O'Connor et al., 2002, *Nature Neurosci.*, 5(9): 823-824). Also the downregulation of synaptotagmin expression in cholinergic neurons of the nucleus basalis in patients with Alzheimer's disease was reported (Mufson et al., 2002, *Neurochem. Res.*, 27(10): 1035-1048), this downregulation is highly specific, as no downregulation was observed for synapsin I, synaptobrevin or SNAP-29 in the same study.

Like synaptotagmin, the SLP and Slac2 proteins are also believed to play a role in regulation of vesicular trafficking (see Strom et al., 2002, *J. Biol. Chem.*, 277: 25423-25430 and Kuroda et al., *J. Biol. Chem.*, 277: 9212-9218 for a description of the role of SHD containing proteins and their relevance to disease states). The SHD is a binding domain for the GTP-bound form of Rab27a, one of the small GTP-binding proteins that are believed to be essential components of the membrane trafficking mechanism of eukaryotic cells (Zerial et al., 2001, *Nat. Rev. Mol. Cell. Biol.* 2, 107-117). The C-terminal domains of the SLP and Slac2 proteins are likely to play a role in the localization Rab27a to specific sites in a cell.

5 Rab27a is involved in the transport of melanosomes in melanocytes and lytic
granules in cytotoxic T-lymphocytes. Griscelli syndrome, a disease caused by a mutation
in Rab27 which leads to defects in the transport of melanosomes and lytic granules is
characterized by partial albinism and severe immunodeficiency with hemophagocytic
syndrome. Overexpression of the SHD sequence led to a dominant negative effect and a
10 defect in the transport of melanosomes in melanocytes that was similar to that observed
in Griscelli syndrome patients.

Alternative splicing occurs at the C2 domain locus and variants of the
synaptotagmin-like proteins have been identified. Additional splice variants have been
suggested but supporting sequence confirmation is not yet available. Also a gene
15 encoding a synaptotagmin-like protein contains a region of weak similarity to murine
Gph.

High Mobility Group Chromosomal Protein HMG17 (also known as HMGN2) is
a member of the HMG 14/17 (also known as HMGN) family of proteins; which bind
DNA with low specificity and share a common DNA-binding motif with members of the
20 HMG 1/2 (also known as HMGB) family of proteins.

Chromosomal proteins HMG-17 and HMG-14 are among the most abundant,
ubiquitous, and evolutionarily conserved nonhistone proteins found in the nuclei of
higher eukaryotes (Landsman et al., 1986, *J. Biol. Chem.*, 261(16): 7479-7484). A large
number of retropseudogenes are scattered over several chromosomes. It has been shown
25 that the nonhistone chromosomal proteins HMG-14 and HMG-17 are encoded by distinct
genes, each of which is part of a separate multigene family. These families may have
evolved independently from similar genetic elements or from a shared ancestral gene in

5 which the nucleotide sequence coding for the DNA-binding domain of the protein is the
most conserved region. The structural differences between the molecules and the
differences in their DNA-binding domains suggest that the proteins may be involved in
distinguishable cellular functions. It was suggested that they may confer specific
conformations to transcriptionally active regions of chromatin (Weisbrod and Weintraub,
10 1979, *Proc. Natl. Acad. Sci. USA*, 76: 630) thereby changing the transcriptional potential
of the chromatin template (Almouzni et al., 1990, *EMBO J.*, 9: 573; Svaren and Chalkley,
1990, *Trends Genet*, 6: 52). Microinjections of antibodies to HMG-17 into human
fibroblasts inhibited transcription (Einck and Bustin, 1983, *Proc. Natl. Acad. Sci. USA*,
80: 6735). Some data suggest that HMG-17 binds to chromatin in a tissue specific
15 manner (Brotherton et al., 1990, *Nucl. Acids Res.*, 18: 2011).

The putative role of HMG-17 in chromatin structure and gene expression is
supported by its differential expression during cell differentiation (reviewed in Bustin et
al., 1992, *CRC Crit. Rev. Eukaryotic Gene Expression*, 2: 137). Analyses of the mRNA
levels during the course of erythropoiesis (Crippa et al., 1991, *J. Biol. Chem.*, 266: 2712),
20 myogenesis (Pash et al., 1990, *J. Biol. Chem.*, 265: 4197), osteoblast differentiation of
several additional cell lines (Crippa et al., 1990, *Cancer Res.*, 50: 2022) indicate that
undifferentiated cell synthesize more HMG mRNA than differentiated cells. Results
suggest that myogenic differentiation may require regulated levels of HMG-14 (Pash et
al., 1993, *J. Biol. Chem.*, 268: 13632) and that HMG-14 mRNA and protein levels are
25 elevated in tissues from the individuals with Down syndrome (Pash et al., 1991, *Exp. Cell*
Res., 193: 232) and in trisomy-16 mouse (Pash et al., 1993, *J. Biol. Chem.*, 268: 13632).

5 A 31-amino acid synthetic peptide from HMG17, when injected intravenously, accumulates in the nuclei of tumor endothelial cells and tumor cells and can carry a “payload” such as a fluorescent label to a tumor and into the cell nuclei in the tumor. This result suggests that HMGN2, like HMGB1, may have a role as an extra-cellular signaling molecule (Porkka et al., 2002, *Proc. Natl. Acad. Sci. USA*, 99: 7444-7449).

10 PIN2-interacting protein 1 (PinX1) is an RNA processing protein, which contains a G-patch domain (Aravind and Koonin, 1999, *Trends Biochem Sci.*, 24: 342-344). The 328-amino acid length of PinX1 protein is predicted from a longest polynucleotide sequence of PINX1 identified in a yeast 2-hybrid assay. The protein contains no known domain structure except for a gly-rich region in its N-terminus and a telomerase (TERT) inhibitory domain (TID) in its C terminus. The prediction is confirmed by a Northern blot analysis which has detected a 1.9-kb PINX1 transcript in all tissues tested. Immunoprecipitation and immunoblot analyses indicate that PINX1 encodes a 45-kD protein in cells (Zhou and Lu, 2001, *Cell*, 107: 347-359).

 PinX1 is identified as a Pin2 binding protein in a yeast 2-hybrid assay and confirmed in coimmunoprecipitation, colocalization and pull-down experiments (Zhou and Lu, 2001, *Cell*, 107: 347-359). It is also discovered that PINX1 inhibits telomerase activity and affect tumorigenicity, when the small TID domain of PinX1 binds the telomerase catalytic subunit hTERT and potently inhibits hTERT activity (Zhou and Lu, 2001, *Cell*, 107: 347-359; Kishi and Lu, 2002, *J. Biol. Chem.*, 277(9): 7420-7429).

25 Telomerase activity is important for normal and transformed human cells and is implicated in oncogenesis. Overexpression of PinX1 or its TID domain inhibits telomerase activity, shortens telomeres, and induces crisis, whereas depletion of

5 endogenous PinX1 increases telomerase activity and elongates telomeres. Depletion of
PinX1 also increases tumorigenicity in nude mice, consistent with its chromosome
localization at 8p23, a region with frequent loss of heterozygosity in a number of human
cancers. Thus, PinX1 is a potent telomerase inhibitor and a putative tumor suppressor
(Zhou and Lu, 2001, *Cell*, 107: 347-359; Kishi and Lu, 2002, *J. Biol. Chem.*, 277(9):
10 7420-7429).

Unlike human PinX1, which inhibits telomerase activity, the putative yeast
homolog of PinX1, encoded by the YGR280c open reading frame (ORF), is a component
of the ribosomal RNA processing machinery involved in rRNA and small nucleolar RNA
maturation (Guglielmi and Werner, 2002, *J. Biol. Chem.*, 277(38): 35712-35719). The
15 protein has a KK(E/D) C-terminal domain typical of nucleolar proteins and a putative
RNA interacting domain widespread in eukaryotes called the G-patch. The protein is
hence renamed Gno1p (G-patch nucleolar protein). GNO1 deletion results in a large
growth defect due to the inhibition of the pre-ribosomal RNA processing first cleavage
steps at sites A(0), A(1), and A(2). Furthermore, Gno1p is involved in the final 3'-end
20 trimming of U18 and U24 small nucleolar RNAs. Mutational analysis shows that the G-
patch of Gno1p is essential for both functions, whereas the KK(E/D) repeats are only
required for U18 small nucleolar RNA maturation.

Human PinX1 expression in the yeast multicopy expression vector pGen in the
background of gno1-Delta (deletion of GNO1 gene) phenotype suggests that it has a dual
25 function in telomere length regulation and ribosomal RNA maturation in agreement with
its telomeric and nucleolar localization reported in human cells (Guglielmi and Werner,

5 2002, *J Biol Chem*, 277(38): 35712-35719). Conversely, the same study finds that a full length yeast Gno1p does not exhibit the *in vivo* telomerase inhibitory activity of PinX1.

 CBF1-interacting corepressor (CIR) is a unique CBF1 interacting corepressor, which binds to histone deacetylase and to SAP30 and serves as a linker between CBF1 and the histone deacetylase complex (Hsieh et al., 1999, *Proc Nat Acad Sci*, 96: 23-28).

10 CBF1 (RBPSUh) is a member of the CSL family of DNA-binding factors, which mediate transcriptional activation or repression. The family includes CBF1, 'suppressor of hairless,' and Lag1 (Schweisguth and Posakony, 1992, *Cell*, 69: 1199-1212; Christensen et al., 1996, *Development (Cambridge, UK)*, 122: 1373-1383). CSL proteins play a central role in Notch signaling (Artavanis-Tsakonas et al., 1995, *Science*, 268: 225-232).

15 Disruptions and aberrations in Notch signaling are associated with human neoplastic disease, cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencepholopathy, and Alagille syndrome. CSL proteins also play a central role in and in Epstein-Barr virus-induced immortalization (Zhou et al., 2000, *J. Virol.*, 74(4): 1939-1947; Hsieh et al., 1999, *Proc. Natl. Acad. Sci. USA*, 96: 23-28 and references
20 therein), the process associated with human malignancies such as Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease and lymphoproliferative disease in immunosuppressed patients. CIR is believed to play an important role in the gene regulation activity of CBF1 by targeting CBF1 recognition sequences for histone deacetylation, an activity which is linked to gene suppression.

25 Human CIR is a 450-amino acid protein (560-amino acid *C. elegans* homolog) with a highly charged, serine-rich C-terminus predicted from a polynucleotide sequence identified using a yeast 2-hybrid screen of a B-cell cDNA library with CBF1 as bait

5 (Hsieh et al., 1999, *Proc. Natl. Acad. Sci. USA*, 96: 23-28). The widespread expression of CIR is revealed by Northern blot analysis, with strongest expression detected in heart, skeletal muscle, and pancreas. Immunofluorescence analysis shows that CIR is a nuclear protein like CBF1, although CIR does not bind to the nucleolus. It is determined that the N-terminal 121 amino acids of CIR interact with amino acids 233 to 249 of CBF1 and
10 repress transcriptional activity. Yeast two-hybrid assay and immunofluorescence analysis indicate that CIR also interacts with HDAC2 and SAP30, important mediators of transcriptional repression.

Cullin 3, a member of the cullin/Cdc53 family of proteins, is a putative E3 ubiquitin ligase which regulates abundance of other proteins, such as Ci (Ou et al., 2002,
15 *Genes Dev.*, 16(18): 2413-2414), cyclins E and D1 (Singer et al., 1999, *Genes Dev.*, 13: 2375-2387; Winston et al., 1999, *Genes Dev.*, 13: 2351-2357; Maeda et al., 2001, *FEBS Lett.*, 494: 181-185) and katanin (Kurtz et al., 2002, *Science*, 295: 1294-1298) by targeting them for ubiquitin-dependent proteolysis, potentially as a part of a larger complex.

20 Ci protein is required for cell's ability to sense and interpret graded spatial information and therefore ultimately responsible for cell's fate (Ou et al., 2002, *Genes Dev.*, 16(18): 2413-2414; Jiang J, 2002, *Genes Dev.*, 16: 2315-2321). Overexpression of cyclin D1 has been implicated in a variety of tumors such as breast cancers, gastrointestinal cancers and lymphomas (Maeda et al., 2001, *FEBS Lett.*, 494: 181-185).
25 Cyclin E is an evolutionary conserved protein whose essential function is to promote cell cycle transition from G1 to S thereby coordinating crucial events in the organism (Knoblich et al., 1994, *Cell*, 77: 107-120; Ohtsubo et al., 1995, *Mol. Cell. Biol.*, 15: 2612-

5 2624). Katanin, which has documented microtubule-severing activity, regulates microtubule instability and the loss of katanin completely suppresses all signs of instability (Han et al, 1997, *Nature*, 386: 296; Kurtz et al., 2002, *Science*, 295: 1294-1298) in *C. elegans*.

The cullins represent a conserved gene family, with at least five members in
10 nematodes, six in humans, and three in *S. cerevisiae*. Human CUL3 is an ortholog of nematode cul3 (Winston et al., 1999, *Genes Dev.*, 13: 2351-2357) which is expressed in several tissues as major 2.8- and minor 4.3-kb transcripts in various tissues, with the highest levels in skeletal muscle and heart. CUL3 has been identified as a gene whose expression in human fibroblasts is induced by phorbol 12-myristate 13-acetate (PMA)
15 and suppressed by salicylate. Homozygous deletion of the Cul-3 gene is shown to cause embryonic lethal phenotype (Singer et al., 1999, *Genes Dev.*, 13: 2375-2387).

The methods developed for monitoring the ubiquitylation activity of cullins, or modulating such activity are described in U.S. Patent No. 6,426,205 to Tyers et al., and U.S. Patent Nos. 6,165,731 and 6,413,725 to Deshaies et al., hereby incorporated by
20 reference.

High-mobility group protein HMGN3, also known as a thyroid hormone receptor interacting protein 7 (Trip7), is a member of a class of relatively abundant non-histone nuclear proteins, which function as architectural elements (Bustin and Reeves, 1996, In Cohn, Moldave eds., *Progress in Nucleic Acid Research and Molecular Biology*, Vol. 54,
25 San Diego, Academic Press, 35-100). HMGN3 (Trip7) is closely related to the small nonhistone chromatin proteins HMG14 and HMG17 (HMGN2), which bind specifically to nucleosomes, reduce the compactness of chromatin fiber and enhance transcription

5 from chromatin templates (Bustin, 2001, *Trends Biochem. Sci.*, 26: 431-437). In addition, a yeast two-hybrid screen in a HeLa cell library indicated that HMGN3 interacts with the ligand binding domain of thyroid hormone receptor beta (TR β ₁), but only in the presence of thyroid hormone (Lee et al., 1995, *Mol. Endocrinol.*, 9: 243-254).

The thyroid hormone receptors (TRs) are hormone-dependent transcription factors
10 that regulate expression of a variety of specific target genes. It is suggested that they are regulated by a number of proteins as they progress from their initial translation and nuclear translocation to heterodimerization with retinoid X receptors (RXRs) and further to functional interactions with other transcription factors and the basic transcriptional apparatus, and eventually, degradation (Collingwood et al., 1999, *J. Mol. Endocrinol.*, 23:
15 255-275). Interestingly, all Trips (including HMGN3) interact with RXR-alpha (RXRA) in a ligand-dependent manner, but HMGN3 does not interact with the glucocorticoid receptor (NR3C1) under any conditions (Lee et al., 1995, *Mol. Endocrinol.*, 9: 243-254).

Northern blot analysis detects a 1.1-kb TRIP7 transcript in several tissues, with highest expression in heart and kidney (Lee et al., 1995, *Mol. Endocrinol.*, 9: 243-254).
20 Both Northern and Western analysis demonstrate a tissue specific expression pattern in mice with the highest level of expression in whole mouse brain extracts (West et al., 2001, *J. Biol. Chem.*, 276: 25959-25969). The additional immunohistochemical data reveals that the expression of HMGN3 is enriched in specific regions of the mouse brain with relatively high expression in lateral olfactory tract, anterior commissure, corpus
25 callosum, internal capsule, fornix, stria medullans, optic tract and axon bundles (Ito and Bustin, 2002, *J. Histochem. Cytochem.*, 50(9): 1273-1275). The expression pattern most closely resembles the expression pattern of GFAP (glial fibrillary acidic protein) which is

5 considered an important factor in astrocyte differentiation and is part of the reactive response of the CNS to injury (Eng et al., 2000, *Neurochem. Res.*, 25: 1439-1451). The results raise the possibility that HMGN3 protein plays a functional role in the astrocytes of mouse brain (Ito and Bustin, 2002, *J. Histochem. Cytochem.*, 50(9): 1273-1275).

A separate study of an HMGN3 (Trip7) homolog in *Xenopus laevis* implicated the
10 protein in tissue remodeling during the metamorphosis. The study shows that HMGN3 influences basal transcription in a chromatin structure-dependent manner, but enhances the function of liganded TR regardless of the chromatin structure of the promoter (Amano et al., 2002, *Developmental Dynamics*, 223: 526-535).

HSPC144 protein is a homolog of the chicken thymocyte protein (cThy28), a
15 protein that is suggested to mediate avian lymphocyte apoptosis (Compton et al., 2001, *Apoptosis*, 6: 299-314). Avian cThy28 gene is a 1070 bp cDNA encoding a 242 amino acid conserved protein, cThy28 (GenBank accession number U34350) that shares greater than 90% amino acid similarity with several putative mammalian homologues such as mouse mThy28 (226 aa) (Miyaji et al., 2002, *Gene*, 297: 189-196) and a human
20 HSPC144 (225 aa) obtained from a human CD34⁺ stem cell library. A structural analysis of the protein suggests that it is a nuclear-localized phosphoprotein with potential glycosylation and myristolation sites. Compared to other non-lymphoid tissues, the avian cThy28 protein and its transcript are present in immune organs at elevated levels. The mouse homolog is expressed in testis, liver, brain and kidney with the lower levels of
25 expression in thymus spleen, heart and stomach (Miyaji et al., 2002, *Gene*, 297: 189-196). The high degree of conservation in amino acid sequences among various species

5 including bacteria, yeast and plants as well as vertebrate suggests an indispensable role of the protein in living cells.

Human Cell Cycle Controller CDC6 protein, a protein that is highly similar to *Saccharomyces cerevisiae* Cdc6 protein, is essential for the initiation of DNA replication (for review see, Bell and Dutta, 2002, *Annu. Rev. Biochem.*, 71: 333-374; Lee and Bell, 10 2000, *Curr. Opin. Cell Biol.*, 12: 280-285; Lei and Tye, 2001, *J. Cell Sci.*, 114: 1447-1454; Coleman, 2002, *Curr. Biol.*, 12(22): R759). Cdc6 is part of a macromolecular machine that assembles on chromatin to target the DNA for a single round of replication. Cdc6 (*Saccharomyces cerevisiae*) and Cdc18 (*Schizosaccharomyces pombe*) collaborate with the six-subunit origin recognition complex (ORC, Orc1) (Cocker et al., 1996, 15 *Nature*, 379: 180-182; Tanaka et al., 1997, *Cell*, 90: 649-660), Cdt1 (Maiorano et al., 2000, *Nature*, 404: 622-625; Nishitani et al., 2000, *Nature*, 404: 625-628) and with the DNA replication proteins (PCNA, RPA) to recruit minichromosome maintenance (MCM) family of proteins to DNA, thereby forming the pre-replication complex.

Human CDC6 localizes in cell nucleus during cell cycle G1, but translocates to 20 the cytoplasm at the start of S phase, suggesting that DNA replication may be regulated by either the translocation of this protein between the nucleus and cytoplasm or by selective degradation of the protein in the nucleus, as revealed by immunofluorescent analysis of epitope-tagged protein (Delmolino et al., 2001, *J. Biol. Chem.*, 276(29): 26947-26954). The subcellular translocation of Cdc6 during cell cycle is also regulated 25 through its phosphorylation by Cdks at nuclear localization signals or at nuclear export signals or at sites adjacent to these (Delmolino et al., 2001, *J. Biol. Chem.*, 276(29): 26947-26954).

5 There is evidence that human Cdc6 regulates the onset of mitosis, as
overexpression of human Cdc6 in G2 phase cells prevents entry into mitosis by blocking
cells in G2 phase via a checkpoint pathway involving Chk1 (Clay-Farrace et al., 2003,
EMBO J., 22(3): 704-712). Transcription of human Cdc6 is regulated in response to
mitogenic signals through transcriptional control mechanism involving E2F proteins, as
10 revealed by a functional analysis of the human Cdc6 promoter and by the ability of
exogenously expressed E2F proteins to stimulate the endogenous Cdc6 gene (Ohtani et
al., 1998, *Oncogene*, 17: 1777-1785; Hateboer et al., 1998, *Mol. Cell. Biol.*, 18(11): 6679-
6697).

Northern blots indicate that CDC6/Cdc18 mRNA levels peak at the onset of S
15 phase and diminish at the onset of mitosis in HeLa cells, but total CDC6/Cdc18 protein
level is unchanged throughout the cell cycle. Immunoprecipitation studies show that
human CDC6/Cdc18 associates *in vivo* with cyclins (such as cyclin A and B), CDK 1
and/or CDK2, and ORC1 (Clay-Farrace et al., 2003, *EMBO J.*, 22(3): 704-712; Yam et
al., 2002, *Cell Mol. Life Sci.*, 59: 1317-1326). The association of cyclin-CDK2 with
20 CDC6/Cdc18 is specifically inhibited by a factor present in mitotic cell extracts. It has
been suggested that if the interaction between CDC6/Cdc18 with the S phase-promoting
factor cyclin-CDK2 is essential for the initiation of DNA replication, the mitotic inhibitor
of this interaction could prevent a premature interaction until the appropriate time in G1
(for review see, Yam et al., 2002, *Cell Mol. Life Sci.*, 59: 1317-1326).

25 Cdc6 is expressed selectively in proliferating but not quiescent mammalian cells,
both in culture and within tissues in intact animals. For example, nuclear human Cdc6 is
detected by immunofluorescence in 90% of nuclei in premalignant human cervical tissue

5 (Williams et al., 1998, *Proc. Natl. Acad. Sci. USA*, 95: 14932-14937). Most studies agree
that markers of proliferation correlate with patient prognosis (Gerdes et al., 1983, *Intl. J.*
Cancer, 31: 13-20; Thapar et al., 1996, *Neurosurgery*, 38: 99-106). The expression of
Cdc6 may be used as marker of proliferating cells in various types of tumors (Freeman et
al., 1999, *Clin. Cancer. Res.*, 5: 2121-2132; Ohta et al., 2001, *Oncology Reports*, 8: 1063-
10 1066). During the transition from a growth-arrested to a proliferative state, transcription
of mammalian Cdc6 is regulated by E2F proteins (Ohtani et al., 1998, *Oncogene*, 17:
1777-1785). For example, a significant downregulation of Cdc6 expression found in
prostate cancer is attributed to E2F and Oct1 transcription factors (Robles et al., 2002, *J.*
Biol. Chem., 277(28): 25431-25438). Immunodepletion of Cdc6 by microinjection of
15 anti-Cdc6 antibody blocks initiation of DNA replication in human tumors including
tumors of neuroepithelial tissue, vestibular schwannomas, meningiomas and plurality
adenomas, or human tumor cell lines (Ohta et al., 2001, *Oncology Reports*, 8: 1063-
1066).

The additional regulatory event controlling initiation of DNA replication in
20 mammalian cells is hypothesized to be dephosphorylation of CDC6 by PP2A, mediated
by a specific interaction with PR48 or a related B'' protein (Yan et al., 2000, *Mol. Cell*
Biol., 20(3): 1021-1029). The study demonstrates that an N-terminal segment of CDC6
binds specifically to PR48, a regulatory subunit of protein phosphatase 2A (PP2A).

25 4. SUMMARY OF THE INVENTION

Controlling the levels of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3,
HMGN3, HSPC144 and CDC6 is important in maintaining proper health and improper

5 control of these levels is cause for disease. Applicants have discovered that these proteins are targets for degradation via the N-end rule ubiquitylation pathway. Controlling the rate of this degradation pathway provides an important mechanism for modulating the levels of these proteins and, thus, controlling disease states that are affected by the levels of these proteins.

10 One embodiment of the invention is a complex comprising at least one ubiquitin, or a derivative thereof, and a protein, wherein said protein is selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof. The complex is formed via the N-end rule ubiquitylation. Preferably, the complex is present *in vitro* and/or has been prepared
15 via an *in vitro* ubiquitylation reaction. Preferably the complex is enriched or isolated relative to the conditions in which it is found *in vivo*. More preferably, the complex has a specific activity that is at least five-fold greater than the specific activity of an unenriched complex in solubilized cell lysates, cells or tissues. The complex may be immobilized on a solid support and/or linked to a label.

20 The invention also includes methods for producing these complexes, the methods comprising forming a mixture comprising i) a vector containing an insert coding for a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6; ii) an *in vitro* translation system and iii) an N-end rule ubiquitylation system and incubating the mixture to allow formation of the
25 complex. Preferably, the *in vitro* translation system is also capable of carrying out *in vitro* transcription. Especially preferred *in vitro* translation systems comprise eucaryotic cell lysate, more preferably reticulocyte lysate, most preferably rabbit reticulocyte lysate.

5 The lysates may be supplemented with, e.g., RNA polymerases to catalyze transcription and substrates and/or regulators of the transcription/translation reactions such as amino acids, tRNAs, ATP, etc. Preferably, the mixture includes a proteasome inhibitor. The method may further comprise the step of isolating the complex, preferably by binding the complex to an antibody specific for the protein or an antibody specific for poly-ubiquitin
10 chains. Most preferably, the production of the complex is inhibited by the addition of an inhibitor of the N-end rule pathway.

The invention also includes an isolated activated fragment of a protein having an exposed N-degron, wherein said protein is selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6,
15 fragments and derivatives thereof. Preferably, the activated fragment is present *in vitro* and/or has been prepared via an *in vitro* reaction. Preferably the activated fragment is enriched or isolated relative to the conditions in which it is found *in vivo*. Most preferably, the activated fragment has an activity as a substrate for N-end rule ubiquitinylation, e.g., as measured in a ubiquitylation assay, that is at least five-fold
20 greater than the activity of an equivalent concentration of the corresponding whole protein. The activated fragment may be immobilized on a solid support and/or linked to a label.

The invention also includes a method of producing these activated fragments, the method comprising forming a mixture comprising i) a, preferably isolated and/or
25 enriched, protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6; and ii) one or more enzymes (preferably, a protease) that modify said protein to form an N-degron; and incubating the

5 mixture to allow production of the activated fragment. Optionally, the protein is prepared by *in vitro* translation or transcription/translation. In one exemplary embodiment, the method comprises forming a mixture comprising i) a vector containing a clone coding for a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6; ii) an *in vitro* translation system that comprises
10 or is supplemented with one or more enzymes that modify the protein to expose an N-degron and incubating the mixture to form the activated fragment. Preferably, the *in vitro* translation system is also capable of carrying out *in vitro* transcription. Especially preferred *in vitro* translation systems comprise eucaryotic cell lysate, more preferably reticulocyte lysate, most preferably rabbit reticulocyte lysate. The lysates may be
15 supplemented with, e.g., RNA polymerases to catalyze transcription and substrates and/or regulators of the transcription/translation reactions such as amino acids, tRNAs, ATP, etc. Preferably, the mixture includes an inhibitor of N-end rule ubiquitylation and/or a protease inhibitor. The method may further comprise the step of isolating the activated fragment.

20 In an alternate embodiment, the activated fragment is formed by a method comprising forming a mixture comprising i) a complex comprising at least one ubiquitin or a derivative thereof, and a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof and ii) a deubiquitylation system and incubating the mixture to allow
25 production of said activated fragment. Preferably, the complex is formed by *in vitro* translation (and, optionally, transcription) as described above. The method may further comprise the step of isolating the activated fragment.

5 The invention also includes an assay composition comprising at least one ubiquitin or a derivative thereof and a protein, wherein said protein is selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof. Preferably, the ubiquitin and/or the protein is immobilized on a support and/or linked to a label. Most preferably
10 one of the ubiquitin or the protein is immobilized on a support and the other is linked to a label.

 The invention also includes a method for identifying peptides having an exposed N-degron which are formed by the action of a protease on a protein, preferably a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3,
15 HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof. The method comprises i) forming a first mixture comprising the protein and a protease that cleaves the protein to form a peptide having an exposed N-degron, and incubating the mixture so as to allow the protease to cleave the peptide to expose the N-degron; ii) forming a second mixture comprising the protein, the protease, and an N-end rule ubiquitylation system,
20 and incubating the mixture so as to allow the protease to cleave the peptide to expose the N-degron and the N-end rule ubiquitylation system to ubiquitylate the peptide and iii) comparing the peptides present in the first and second mixtures to identify the peptides (e.g., by binding assays for ubiquitylated materials, electrophoresis, chromatography, mass spectrometry and/or peptide sequencing) that are non-ubiquitylated in the first
25 mixture and ubiquitylated in the second mixture. Optionally, the N-end rule ubiquitylation system is present in both mixtures but an inhibitor of the system is present in the first mixture to inhibit the system. Optionally, the protein is generated *in situ* by *in*

5 *in vitro* translation (and, optionally, transcription) as described above. Preferably, the translation system also comprises the N-end rule ubiquitylation machinery and/or the protease.

The invention also includes an alternative method for identifying peptides having an exposed N-degron which are formed by the action of a protease on a protein,
10 preferably a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof. The method comprises i) forming a first mixture comprising the protein and a protease that cleaves the protein to form a peptide having an exposed N-degron, and incubating the mixture so as to allow the protease to cleave the peptide to expose the N-
15 degron; ii) forming a second mixture comprising the protein, the protease, an N-end rule ubiquitylation system and a proteasome system, and incubating the mixture so as to allow the protease to cleave the peptide to expose the N-degron, the N-end rule ubiquitylation system to ubiquitylate the peptide and the proteasome system to degrade the peptide and
20 iii) comparing the peptides present in the first and second mixtures to identify the peptides (e.g., by electrophoresis, chromatography, mass spectrometry and/or peptide sequencing) that are present in the first mixture and degraded in the second mixture.

Optionally, the N-end rule ubiquitylation system and/or proteasome systems are present in both mixtures but an inhibitor of one or both systems is present in the first mixture to inhibit ubiquitylation and/or degradation. Optionally, the protein is generated *in situ* by
25 *in vitro* translation (and, optionally, transcription) as described above. Preferably, the translation system also comprises the protease, N-end rule ubiquitylation system and/or the proteasome system.

5 The invention also includes a method for identifying a protease cleavage site which exposes an N-degron in a protein, preferably a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof. The method comprises forming a mixture comprising the protein and a protease which cleaves the protein at said cleavage
10 site, incubating the mixture to allow the protease to cleave at the cleavage site and analyzing the cleavage products to determine the location of the cleavage site. The protein is, preferably, labeled, most preferably with a radioisotope. The location of the cleavage site is, preferably, determined by electrophoretic analysis, chromatography, protein sequencing and/or mass spectrometry. Optionally, the protein is generated *in situ*
15 by *in vitro* translation (and, optionally, transcription) as described above. Preferably, the mixture comprises an inhibitor of N-end rule ubiquitylation and/or a proteasome inhibitor, most preferably an E3 ligase inhibitor. The inclusion of the N-end rule inhibitor is particularly advantageous when one component of the mixture comprises N-end rule ubiquitylation machinery, e.g., if the protease is present in a complex biological
20 sample such as a cell lysate that comprises N-end ubiquitylation machinery.

 The invention also includes a method for identifying proteases which cleave a protein so as to expose an N-degron, the protein preferably selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof. The method comprises i) forming a
25 mixture comprising said protein and a putative protease and ii) incubating the mixture and iii) determining if the protein has been cleaved to form an activated fragment with an exposed N-degron. Preferably, the method further comprises repeating the method with

5 one or more other putative proteases. Even more preferably, a library of putative proteases is screened using the steps of the method. The putative proteases are, optionally, generated by *in vitro* translation and/or transcription.

In one preferred embodiment, determining if the protein has been cleaved comprises assaying for a previously identified activated fragment. In another preferred
10 embodiment, the determining if the protein has been cleaved comprises treating the products of the incubation with an N-end rule ubiquitinylation system and/or a proteome system and identifying proteolytic products that are ubiquitinylated by the ubiquitinylation system and/or degraded by the proteosome system (see, e.g., the methods for identifying for identifying peptides having an exposed N-degron above).

15 The invention also includes a method for identifying E3 ligases comprising combining a putative E3 with an N-end rule substrate; and measuring the binding of the putative E3 to said N-end rule substrate. In one embodiment, the steps of the method are repeated with one or more additional putative E3 ligases to screen a library of putative E3 ligases. Optionally, the putative E3 ligase(s) are produced by *in vitro* translation and/or
20 transcription. Preferably, the N-end rule substrate is a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof. More preferably, the N-end rule is an activated fragment of a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 having an exposed N-
25 degron.

The invention also includes a method for identifying E3 ligases comprising combining a putative E3 with an N-end rule substrate and active ubiquitylation system,

5 preferably in the presence of a proteasome inhibitor and measuring ubiquitylation and/or Ub-dependent degradation of said N-end rule substrate. Optionally, the putative E3 ligase(s) are produced by *in vitro* translation and/or transcription. Preferably, the activity of endogenous E3 ligases in said ubiquitylation system is at least two fold less than the activity of the E3 activity of a putative E3 ligase; more preferably, the ubiquitylation
10 system lacks an active endogenous E3 ligase or is supplemented with inhibitors of the endogenous E3 activity. Preferably, the N-end rule substrate is a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof. More preferably, the N-end rule is an activated fragment of a protein selected from the group consisting of aprataxin,
15 tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof having an exposed N-degron.

The invention also includes a method for identifying one or more active compounds that modulate (promote or inhibit) N-end rule dependent ubiquitylation of a N-end rule substrate, preferably a protein selected from the group consisting of aprataxin,
20 tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof. The method comprises i) forming a mixture comprising the substrate, an N-rule ubiquitylation system and one or more candidate compounds (e.g., compounds selected from a natural product library, a synthetic compound library, a combinatorial compound library and/or a library of FDA approved drugs, etc.); ii)
25 measuring the ubiquitylation and/or degradation of said substrate; and iii) identifying active compounds that modulate the N-end rule dependent ubiquitylation of the N-end rule substrate. Preferably, the active compounds modulate E1 activity, E2 activity, E3

5 activity and/or the activity of a protease that exposes an N-degron. In one embodiment, said substrate is an activated fragment of the protein that comprises an exposed N-degron. In an alternative embodiment, the substrate includes a hidden N-degron and the mixture of step a) further includes a protease which exposes the N-degron. In another embodiment, the method is conducted with a substrate having a hidden N-degron and
10 repeated with the corresponding substrate having an exposed N-degron to determine if an active compound inhibits ubiquitylation or a protease that exposes the N-degron. In yet another embodiment, the method is repeated in the presence of inhibitors of Type I, Type II and/or Type III N-end rule ubiquitylation to determine if an active compound is specific for one of those activities.

15 The invention also includes a method of making a pharmaceutical formulation containing one or more active compounds which modulate an N-end rule ubiquitylation, preferably of a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6. The method comprises the steps of identifying the one or more active compounds as described above and
20 incorporating the one or more compounds into a pharmaceutical formulation comprising the at least one active compounds and a suitable carrier.

The invention also includes a method for modulating N-end rule ubiquitylation of a protein, preferably of a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6, comprising
25 administering one or more of an active compounds that modulates the N-end rule ubiquitylation. Preferably, the active compound is selected as described above.

5 The invention also includes a method for modulating the *in vivo* level of a protein, preferably of a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6, comprising administering one or more active compounds that modulates the N-end rule ubiquitylation. Preferably, the active compound is selected as described above.

10 The invention also includes a method for modulating the *in vitro* or *ex vivo* level of a protein, preferably of a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6, comprising administering one or more of an active compounds that modulates the N-end rule ubiquitylation. Preferably, the active compound is selected as described above.

15 The invention also includes a method for treating a disease comprising administering one or more of an active compounds that modulates the N-end rule ubiquitylation of a protein, preferably of a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6. Preferably, the active compound is selected as described above.

20 The invention also includes a method of changing the susceptibility of a protein to N-end rule ubiquitylation by modifying the protein (e.g., by mutating the gene encoding the protein). The method may comprise i) modifying a protein having a protease cleavage site whose cleavage exposes an N-degron so as to form a modified protein that does not comprise the cleavage site (e.g., by modifying the amino acid sequence so it is
25 no longer recognized by a protease which cleaves the unmodified protein at the cleavage site); ii) modifying a protein to introduce a protease cleavage site for a known protease, the cleavage of which exposes an N-degron, so as to form a modified protein that is

5 cleaved by the protease to expose an N-degron; or iii) modifying a protein to alter an exposed N-degron or a hidden N-degron so as to form a modified protein that is not recognized by an N-end rule E3 ligase (in the case of hidden N-degrons, this method includes changing the identity of internal amino acids which are recognized in the unaltered protein by the E3 ligase after proteolytic processing of the protein). The modified protein may be expressed in a cell. Preferably, the cell does not express the wild type protein or the gene for the wild type protein is replaced with the gene for the modified protein. By introducing the modified protein, the level of the protein in the cell is modulated relative to the level of the protein in an analogous cell expressing the wild type protein.

15 The invention also includes a method of modulating the abundance of an N-end rule substrate in a cell by i) mutating the substrate to remove a protease cleavage site that is cleaved to introduce an N-degron; ii) mutating the substrate to introduce a protease cleavage site for a known protease, the cleavage of which exposes an N-degron; or iii) mutating the substrate to alter an N-degron so that it is not recognized by an E3 recognition after protease cleavage. Preferably, the substrate is a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6.

25 The invention also includes a method of generating a phenotypic cell line, or an animal, comprising the steps of i) generating a transfection vector, comprising a clone coding for a mutated form of a protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof, the mutated protein having a mutated protease cleavage site

5 and/or N-degron and thereby modulating the susceptibility of the protein to the N-end rule ubiquitylation; ii) using said vector to transfect a cell line or generate a transgenic animal by homologous or non-homologous recombination, optionally via *in vitro* fertilization. The mutation is selected to modulate the rate at which the protein is cleaved by a, preferably endogenous, protease that exposes an N-degron and/or to modulate the rate of N-end rule ubiquitylation of a, preferably hidden, N-degron. The method, preferably, further comprises the step of detecting phenotypic changes in the cell line or animal relative to a control cell line or animal expressing the non-mutated form of the protein.

The invention also includes a kit for producing an N-end rule ubiquitylated protein, the protein selected from the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof. The kit comprises, in one or more containers, one or more DNA sequences coding for the protein and one or more assay components selected from the group consisting of: (a) RRL; (b) plates; (c) one or more binding reagents; (d) a pH buffer; (e) one or more blocking reagents; (f) antibodies; (g) luminescent label; (h) luminescence co-reactant; (i) preservatives; (j) stabilizing agents; (k) enzymes; (l) detergents; (m) inhibitors and (n) desiccants.

The invention also includes a library of N-end rule ubiquitylation substrates. The library includes at least one, preferably a plurality, more preferably all, of the proteins in the group consisting of aprataxin, tau, SLP, HMG17, PinX1, CIR, Cullin 3, HMGN3, HSPC144 and CDC6 and fragments and derivatives thereof.

5 **5. BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows a schematic representation of three binding assay formats used to measure ubiquitylation of a protein.

Figure 2 shows (A) the time course for an ECL assay for N-end rule ubiquitylation of RGS4; (B) the % inhibition of the ECL signal in the presence of N-end rule inhibitors; and (C) gel electrophoretic analysis of the products of the ubiquitylation reactions.

Figure 3 shows (A) the time course for an ECL assay for N-end rule ubiquitylation of tau; (B) the % inhibition of the ECL signal in the presence of N-end rule inhibitors; (C) gel electrophoretic analysis of the products of the ubiquitylation reactions; and (D) an overexposed image of the gel of Figure 3(C).

Figure 4 shows (A) the time course for an ECL assay for N-end rule ubiquitylation of aprataxin; (B) the % inhibition of the ECL signal in the presence of N-end rule inhibitors; and (C) gel electrophoretic analysis of the products of the ubiquitylation reactions.

Figure 5 shows (A) the time course for an ECL assay for N-end rule ubiquitylation of synaptotagmin-like protein 2; (B) the % inhibition of the ECL signal in the presence of N-end rule inhibitors; and (C) gel electrophoretic analysis of the products of the ubiquitylation reactions.

Figure 6 shows (A) the time course for an ECL assay for N-end rule ubiquitylation of HMGN2; and (B) the % inhibition of the ECL signal in the presence of N-end rule inhibitors.

5 Figure 7 shows (A) the time course for an ECL assay for N-end rule ubiquitylation of CDC6; (B) the % inhibition of the ECL signal in the presence of N-end rule inhibitors; and (C) gel electrophoretic analysis of the products of the ubiquitylation reactions.

 Figure 8 shows (A) the time course for an ECL assay for N-end rule ubiquitylation of HSPC144 and (B) the % inhibition of the ECL signal in the presence of
10 N-end rule inhibitors.

 Figure 9 shows (A) the time course for an ECL assay for N-end rule ubiquitylation of PinX1 and (B) the % inhibition of the ECL signal in the presence of N-end rule inhibitors.

15 Figure 10 shows (A) and (C) the time course for an ECL assay for N-end rule ubiquitylation of the products produced from two clones of HMGN3 and (B) and (D) the % inhibition of the ECL signal from these two clones in the presence of N-end rule inhibitors.

 Figure 11 shows (A) the time course for an ECL assay for N-end rule ubiquitylation of CIR and (B) the % inhibition of the ECL signal in the presence of N-end
20 rule inhibitors.

6. DETAILED DESCRIPTION

 The invention relates to methods for identifying biologically significant proteins,
25 protein interactions, interaction loci and pathways. The terms "protein", "polypeptide" and "peptide" are used interchangeably herein. The invention also relates to the proteins identified by these methods and variants thereof. The invention also relates to products

5 of interactions of these proteins (or variants thereof) with other components of a
biological pathway. The invention also relates to methods of identifying biologically
active substances that modulate the activity of these proteins and the use of these in
pharmaceutical compositions. The invention also relates to diagnostic methods that
involve measuring these proteins or their associated biological activity. Furthermore the
10 invention is related to reagents, kits and assay compositions, which assist in carrying out
the methods of the instant invention.

Applicants have identified novel substrates of N-end rule ubiquitylation. These
substrates include MAPT (tau) (e.g., SEQ ID NO: 1 encoded by polynucleotide sequence
SEQ ID NO: 13 or the protein encoded by IMAGE clone #4448167), aprataxin (e.g.,
15 SEQ ID NO: 2 encoded by polynucleotide sequence SEQ ID NO: 14 or the protein
encoded by IMAGE clone #3994375), synaptotagmin-like protein 2 (SLP2) (e.g., SEQ
ID NO: 3 encoded by polynucleotide sequence SEQ ID NO: 15 or the protein encoded by
IMAGE clone # 3887570), HMG17 (HMGN2) (e.g., SEQ ID NO: 4 encoded by
polynucleotide sequence SEQ ID NO: 16 or the protein encoded by IMAGE clone #s
20 3447081 or 3455121), CDC6 cell cycle controller protein (CDC6) (e.g., SEQ ID NO: 6
encoded by polynucleotide sequence SEQ ID NO 18 or the protein encoded by IMAGE
clone # 3867414), cullin 3 (e.g., SEQ ID NO: 7 encoded by polynucleotide sequence
SEQ ID NO: 19 or the protein encoded by IMAGE clone # 4426807), HMGN3 (e.g.,
SEQ ID NO: 8 encoded by polynucleotide sequence SEQ ID NO: 20 or SEQ ID NO: 9
25 encoded by polynucleotide sequence SEQ ID NO: 21 or the protein encoded by IMAGE
clone #s 3890883 or 4749335), CIR (e.g., SEQ ID NO: 10 encoded by polynucleotide
sequence SEQ ID NO: 22 or the protein encoded by IMAGE clone # 3910222),

5 HSPC144 (e.g., SEQ ID NO: 11 encoded by polynucleotide sequence SEQ ID NO: 23 or
the protein encoded by IMAGE clone # 3907737) and PIN2-interacting protein 1 (PinX1)
(e.g., SEQ ID NO: 12 encoded by polynucleotide sequence SEQ ID NO: 24 or the protein
encoded by IMAGE clone # 3911679) (Table 1). The definition of an N-end rule
ubiquitylation substrate includes: (i) proteins which are ubiquitylated via N-end rule
10 pathway, (ii) proteins which can be modified to form a fragment having an exposed N-
degron, for example, by cleaving an N-terminal portion of a protein and (iii) a fragment
of a protein having an exposed N-degron.

Table 1.

	<i>Protein</i>	<i>Seq IDs for Protein</i>	<i>Seq IDs for Gene</i>	<i>Image Clone #</i>
1	Microtubule Associated Protein Tau (MAPT, tau)	1	13	4448167
2	Aprataxin	2	14	3994375
3	Synaptotagmin-like protein 2 (SLP2)	3	15	3887570
4	HMG17 (HMGN2)	4	16	3447081 or 3455121
5	CDC6 Cell Cycle Control	6	18	3867414
6	Cullin 3	7	19	4426807
7	HMGN3	8, 9	20, 21	3890883 or 4749335
8	CBF1 Interacting Protein	10	22	3910222
9	HSPC144	11	23	3907737
10	PIN2-interacting protein 1	12	24	3911679

5 As is well known, genes may exist in single or multiple copies within the genome
of an individual. Such duplicate genes may be identical or may have certain
modifications, including splice variants, nucleotide substitutions, additions or deletions,
which all still code for polypeptides having substantially the same activity. The term
"sequence encoding an N-end substrate protein" may thus refer to one or more genes
10 within a particular individual. Moreover, certain differences in nucleotide sequences may
exist between individual organisms, which are called alleles. Such allelic differences
may or may not result in differences in amino acid sequence of the encoded polypeptide
yet still encode a polypeptide with the same biological activity.

 The present invention preferably relates to an isolated protein selected from a
15 group listed in Table 1 and/or a protein selected from a group listed in Table 1 produced
in vitro. The term "isolated" as used herein refers to material that is removed from its
natural environment. For example, recombinant proteins produced *in vitro*, preferably in
an *in vitro* protein expression system; recombinant proteins expressed in genetically
modified cells; and/or proteins extracted and/or enriched from natural sources. The term
20 isolated as used herein also includes purified material, for example, material that is
substantially free of additional cellular material, viral material, or culture medium when
produced by recombinant DNA techniques, or chemical precursors or other chemicals
when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include
nucleic acid fragments which are not naturally occurring as fragments and would not be
25 found in the natural state. The term "isolated" as used herein also includes polypeptides
purified by means available to a skilled artisan (for example, by means of
chromatography, centrifugation, continuous flow separation, filtration and/or

5 electrophoresis). The term “isolated” as used herein also refers to molecules enriched in their environment by means of stimulation or overexpression in native or non-native *in vitro* expression systems, cell extracts, cell lines and/or tissues, preferably enriched relative to other proteins in the environment by at least 2-fold, more preferably enriched by at least 5-fold, more preferably enriched by at least 10-fold.

10 One aspect of proteins listed in Table 1 is their ability to act as substrates for N-end rule ubiquitylation. The activity of these proteins as ubiquitylation substrates can be determined by measuring the accumulation of ubiquitilated products, for example, by the ability of the products to bind antibodies directed against ubiquitin.

Therefore the present invention relates, in part, to a protein selected from a group
15 listed in Table 1, wherein the specific activity of the protein in a test solution relative to the specific activity of the same protein in an unenriched test solution from a natural source (such as solubilized cell lysates, cells or tissues) is greater preferably by at least five-fold, more preferably by at least 10-fold, more preferably by at least 100-fold, most preferably by at least 1000-fold. The term “specific activity” as used herein is a ratio of
20 the activity to the total protein concentration in a sample and can be presented in terms of concentration or, alternatively, in the case of reactive species such as enzymes or enzymatic substrates in terms of signal in a functional assay such as an enzymatic assay. The present invention also relates to a protein selected from a group listed in Table 1, wherein the concentration of a protein in a solution provided is greater than the
25 concentration of the same protein in an unenriched test solution from a natural source (such as solubilized cell lysates, cells or tissues) by at least 2-fold, preferably by at least five-fold, more preferably by at least 10-fold, more preferably by at least 100-fold, most

5 preferably by at least 1000-fold. In one specific embodiment of the invention, the concentration of a protein selected from a group listed in Table 1 in a test solution is greater than 2%, preferably greater than 5%, more preferably greater than 10%, more preferably greater than 25%, more preferably greater than 50%, more preferably greater than 75%, more preferably greater than 90%, more preferably greater than 95%, most
10 preferably greater than 98% of the total protein in a test solution.

The term “activated fragment” refers herein to a fragment of a protein, the fragment having an exposed N-degron that is hidden in the whole protein. The present invention also relates to an activated fragment of an N-end rule ubiquitylation substrate and/or variants thereof, preferably an isolated activated fragment of an N-end rule
15 ubiquitylation substrate and/or variants thereof, having exposed N-degron, wherein the N-end rule ubiquitylation substrate is selected from the group listed in Table 1.

Preferably, the fragment of the present invention is a C-terminal fragment of protein which is the result of a specific proteolytic cleavage at a site which exposes a destabilized N-terminal residue, preferably a destabilizing Ile, Glu, His, Tyr, Gln, Asp, Asn, Phe, Leu,
20 Trp, Lys, Arg, Ala, Ser, Thr or Cys, more preferably a destabilizing Glu, Gln, Cys, Arg, Lys, His specific to Type I N-end rule substrate, Leu, Ile, Tyr, Phe, Trp specific for Type II substrate and Ala, Ser and Thr specific for Type III substrate. The activated fragment may be subjected to an additional proteolysis from the C-terminus either preceding or following a cleavage event which exposes an N-degron.

25 Activated fragments of aprataxin include a ~20-21.5 kDa C-terminal fragment of the protein with an exposed destabilized N-terminal residue, preferably a destabilizing Ile, Glu, His, Tyr, Gln, Asp, Asn, Phe, Leu Trp, Lys, Arg, Ala, Ser, Thr or Cys, more

5 preferably a destabilizing Type I residue selected from Glu, Gln, Cys, Arg, Lys, and His. Preferably, the fragment is the product of a specific proteolytic cleavage at a site between residues 150-160 of aprataxin which also forms a ~ 16.5-18kDa N-terminal fragment.

Activated fragments of synaptotagmin-like protein 2 include a C-terminal fragment of the protein with an exposed destabilized N-terminal residue, preferably a
10 destabilizing Ile, Glu, His, Tyr, Gln, Asp, Asn, Phe, Leu Trp, Lys, Arg, Ala, Ser, Thr or Cys, more preferably a destabilizing Type II residue selected from Leu, Ile, Tyr, Phe, and Trp. Preferably, the fragment is the product of a specific proteolytic cleavage at a site between residues 1-50 which also forms a ~ 0.5-5 kDa N-terminal fragment.

Activated fragments of MAPT (tau) include a ~ 15-20 kDa fragment of the
15 protein with an exposed destabilized N-terminal residue, preferably a destabilizing Ile, Glu, His, Tyr, Gln, Asp, Asn, Phe, Leu Trp, Lys, Arg, Ala, Ser, Thr or Cys, more preferably a destabilizing Type I residue selected from Glu, Gln, Cys, Arg, Lys, and His. The fragment is the product of multi-site proteolytic cleavages.

Activated fragments of cdc6 include two protein fragments starting with exposed
20 destabilized N-terminal residues, preferably destabilizing Ile, Glu, His, Tyr, Gln, Asp, Asn, Phe, Leu Trp, Lys, Arg, Ala, Ser, Thr or Cys, more preferably destabilizing Type I residues selected from Glu, Gln, Cys, Arg, Lys, and His. The fragments are the product of multi-site proteolytic cleavages. One of the activated fragments is a peptide with a molecular weight of ~40-45 kDa.

25 A person of ordinary skill in the art will recognize that the present invention relates not only to the specific protein sequences disclosed in the specification, but also to protein variants thereof such as fragments, analogs and/or derivatives. A protein variant

5 of a specific protein sequence preferably retains at least one biological function or activity of the specific protein sequence, for example the ability to be ubiquitinated via the N-end rule pathway or the ability to act as a substrate for a protease that exposes an N-degron, etc.

The variants of the polypeptides according to the present invention may be (i) one
10 in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, (ii) one in which there are one or more modified amino acid residues, e.g., residues that are modified by the attachment of substituent groups, (iii) one in which the polypeptide is an
15 alternative splice variant of the polypeptide of the present invention, (iv) fragments of the polypeptides and/or (iv) one in which the polypeptide is fused with another polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification (for example, His-tag) or visualization (for example GFP). The fragments include polypeptides generated via proteolytic cleavage (including multi-site proteolysis) of an
20 original sequence. Variants may be post-translationally, or chemically modified. Such variants are deemed to be within the scope of those skilled in the art from the teaching herein.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one
25 polypeptide to a sequence of a second polypeptide. Variants are defined to include polypeptide sequences different from the original sequence, preferably different from the original sequence in less than 40% of residues per segment of interest, more preferably

5 different from the original sequence in less than 25% of residues per segment of interest,
more preferably different by less than 10% of residues per segment of interest, most
preferably different from the original protein sequence in just a few residues per segment
of interest and at the same time sufficiently homologous to the original sequence to
preserve the functionality of the original sequence and/or the ability to ubiquitylate via N-
10 end rule pathway. The present invention includes protein sequences that are at least 60%,
65%, 70%, 72%, 74%, 76%, 78%, 80%, 90%, or 95% similar or identical to an amino
acid sequence selected from a group listed in Table 1. Still within the scope of the
invention are preferred segments of interest of the sequences of the present invention
which are comprised of at least 10, 25, 50, 100, 150 or 200 amino acid residues.

15 The proteins of the invention can be post-translationally modified. For example,
post-translational modifications that fall within the scope of the present invention have
been observed in rabbit reticulocyte lysate or wheat germ extract include signal peptide
cleavage, glycosylation, acetylation, isoprenylation, proteolysis myristoylation, protein
folding and proteolytic processing (Glass and Pollard, 1990, Promega Notes 26). Some
20 modifications or processing events require introduction of additional biological
machinery. For example, processing events, such as signal peptide cleavage and core
glycosylation, are examined by adding canine microsomal membranes (Walter and
Blobel, 1983, *Meth. Enzymol.*, 96: 84) or *Xenopus* egg extracts (Zhou, et al. U.S. Patent
No. 6,103,489) to a standard translation reaction.

25 The proteins of the invention may include unnatural amino acids formed by post-
translational modification or by introducing unnatural amino acids during translation. A
variety of approaches are available for introducing unnatural amino acids during protein

5 translation. By way of example, special tRNAs, such as tRNAs which have suppressor properties, suppressor tRNAs, have been used in the process of site-directed non-native amino acid replacement (SNAAR) (Noren et al., 1989, *Science* 244:182-188). In SNAAR, a unique codon is required on the mRNA and the suppressor tRNA, acting to target a non-native amino acid to a unique site during the protein synthesis
10 (WO90/05785). However, the suppressor tRNA must not be recognizable by the aminoacyl tRNA synthetases present in the protein translation system (Bain et al., 1991, *Biochemistry* 30:5411-5421).

In certain cases, a non-native amino acid can be formed after the tRNA molecule is aminoacylated using chemical reactions which specifically modify the native amino
15 acid and do not significantly alter the functional activity of the aminoacylated tRNA (Promega Technical Bulletin No. 182; tRNA^{nscend}™: Non-radioactive Translation Detection System, Sept. 1993). These reactions are referred to as post-aminoacylation modifications. For example, the epsilon-amino group of the lysine linked to its cognate tRNA (tRNA^{LYS}), could be modified with an amine specific photoaffinity label (Krieg et
20 at., 1986, *Proc. Natl. Acad. Sci. USA*, 83:8604-8608).

One embodiment of the present invention relates to a complex of an N-end rule substrate and at least one ubiquitin formed via N-end rule pathway, wherein a substrate protein is selected from proteins listed in Table 1 and/or variants thereof. Preferably this complex is isolated. For example the isolated complex maybe produced *in vitro*,
25 preferably in an *in vitro* protein expression system; *in vivo* by expressing recombinant proteins in genetically modified cells; by extracting and/or enriching from natural sources; and/or by chemical synthesis. More preferably the complex has a specific

5 activity or concentration enrichment of at least two-fold greater than the specific activity and/or concentration of an unenriched complex in a natural source such as solubilized cell lysate, cell or tissue, more preferably of at least 5-fold, most preferably of at least 10-fold.

Another preferred embodiment of the instant invention is an assay composition
10 comprising an N-end rule substrate protein selected from proteins listed in Table 1 or an activated fragment thereof, and/or variants thereof and an ubiquitin. Furthermore, in one preferred embodiment of the present invention the complex, or protein, and/or variants thereof or ubiquitin constituents of the complex, or assay composition are immobilized on a support. Suitable supports include supports known in the art of solid phase binding
15 assays and include surfaces of tubes, multi-well plates, particles (e.g., magnetizable particles), filters, porous membranes, electrodes, slides and chips. One especially preferred solid phase support is a carbon electrode surface, more preferably a surface of a carbon ink electrode, most preferably a surface of a plasma-treated patterned carbon ink electrode. Carbon electrodes are described in U.S. Patent No 6,207,369 and multi-well
20 multi-array plates having integrated carbon electrodes are described in a U.S. Patent Application Nos. 10/185,274 and 10/185,363, each of which is incorporated herein by this reference.

Yet in another specific embodiment of the present invention the protein and/or variants thereof or ubiquitin constituents of the complex or assay composition are linked
25 to a detectable label. Detectable labels utilized herein may comprise a radioisotope, a fluorescent (including fluorescence polarization), phosphorescent, luminescent, chemiluminescent and/or electrochemiluminescent compound, an enzyme, or an enzyme

5 co-factor as a label moiety, including binding species recognized by preferably labeled
binding partner (e.g biotin, streptavidin, epitope tags, affinity tags (e.g., His₆),
fluorescein, hapten, immunogen, GST and/or GFP), preferably an ECL label. Examples
of ECL technology, ECL labels, ECL assays and instrumentation for conducting ECL
assays are described in U.S. Patents Nos. 5,093,268; 5,147,806; 5,324,457; 5,591,581;
10 5,597,910; 5,641,623; 5,643,713; 5,679,519; 5,705,402; 5,846,485; 5,866,434; 5,786,141;
5,731,147; 6,066,448; 6,136,268; 5,776,672; 5,308,754; 5,240,863; 6,207,369 and
5,589,136 and published PCT applications WO99/63347; WO00/03233; WO99/58962;
WO99/32662; WO99/14599; WO98/12539; WO97/36931 and WO98/57154.

Advantageously, the immobilized or labeled proteins of the invention may be
15 used in binding assays, preferably immunoassays, for measuring the proteins themselves
or for measuring the extent of modification of these proteins, e.g., the extent of
ubiquitylation, proteolytic cleavage or degradation, see, e.g., the assay techniques
described in US Patent Application Serial No. 10/238,960, entitled "Methods, Reagents,
Kits and Apparatus for Protein Function", hereby incorporated by reference.

20 The invention provides advantageous formats for production, accumulation and
measurement of ubiquitylated target proteins, preferably proteins ubiquitylated via the N-
end rule pathway, more preferably proteins selected from the group listed in Table 1 and
variants thereof. Another preferred embodiment of the present invention is the method to
produce and isolate an activated fragment of an N-rule ubiquitylation substrate having
25 exposed N-degron and/or variants thereof.

According to one embodiment of the present invention the molecules are
produced by forming a mixture of a target protein (or a protein synthesis system that

5 produces, *in situ*, the target molecule) and an ubiquitylation system, preferably an N-end rule ubiquitylation system. The systems preferably produce target molecules *in vitro*, preferably in an *in vitro* protein expression system, preferably a eukaryotic cell lysate (such as a reticulocyte lysate, most preferably a rabbit reticulocyte lysate). These lysates may be supplemented with, e.g., RNA polymerases to catalyze transcription and
10 substrates and/or regulators of the transcription/translation reactions such as amino acids, tRNAs, ATP, etc. A suitable *in vitro* transcription/translation system based on rabbit reticulocyte lysate is commercially available from Promega. The cell lysate not only provides the machinery for protein synthesis but also provides the enzymes necessary for ubiquitylation and the proteases necessary to expose N-degrons for an N-end rule
15 ubiquitylation. In alternative embodiments, the target proteins are produced *in vivo* by expressing recombinant proteins in genetically modified cells; by extracting and/or enriching from natural sources; and/or by chemical synthesis.

In one preferred embodiment the method involves inserting a clone into an expression vector, preferably a clone selected from the group of polynucleotide
20 molecules encoding proteins listed in Table 1, activated fragments having exposed N-degron, or variants thereof. A plurality of suitable expression vectors are well known to a person of ordinary skill in the art.

The method further comprises culturing the expression vectors in (i) an *in vitro* protein expression system, preferably cell lysate, most preferably in reticulocyte lysate, or
25 (ii) in transformed host-cells or cell-free systems under conditions such that the encoded sequence is expressed and the protein is produced, preferably in the transformed host-cells containing all components necessary to support ubiquitylation, preferably N-end

5 rule ubiquitylation, preferably further containing one or more proteasome inhibitors. Yet
in another specific embodiment of the invention, the protein expression system is
supplemented with additional enzymes of the ubiquitylation system, preferably E3
ligases, most preferably an UBR1 E3 ligase.

In one embodiment of the invention, in order to produce an activated fragment of
10 a protein, preferably a protein listed in Table 1, and/or variant thereof having exposed N-
degron, the system is further supplemented with one or more inhibitors of
ubiquitinylation, preferably E3 ligase inhibitors.

Yet in another preferred embodiment of the present invention proteins
ubiquitylated via N-end rule pathway are treated with deubiquitylating enzymes,
15 preferably UBC enzymes, simultaneously and/or at a later time to produce activated
fragments having exposed N-degron. The method includes forming a mixture comprising
either ubiquitylated protein, or a vector encoding an N-end rule ubiquitylation substrate, a
system containing all components necessary to support an expression of an encoded
polypeptide and a proteolytic cleavage necessary to expose N-degron and one or more
20 deubiquitylating enzymes sufficient to remove polyubiquitin chains from an activated
fragment of an N-end rule substrate protein, fragment, analog or derivative thereof and
incubating it for a time sufficient to produce a desired product.

Yet in another preferred embodiment the protein or variant thereof, ubiquitylated
via N-end rule pathway, is produced alone or as a fusion product, preferably a fusion
25 product which incorporates a tag or a marker, preferably an affinity purification tag, most
preferably a biotinylated Lys into polypeptides. The coding sequence can be cloned in
frame with the sequence encoding a tag, preferably a His-tag, GST-fusion or GFP-fusion

5 or an expression system can be supplemented with biotinylated Lys-tRNA. In one specific embodiment of the invention, the portion fused to an N-end rule ubiquitylation substrate can be cleaved by proteolytic enzymes available to skilled artisan.

The proteins ubiquitylated via N-end rule pathway and/or their activated fragment are further isolated by one or more of the isolation protocols, such as chromatography, centrifugation, continuous flow separation, filtration and/or electrophoresis. The preferred protocols include affinity chromatography using affinity tags incorporated into polypeptides and/or antibodies specific for polyubiquitin chains, streptavidin binding to a product having biotinylated lysines or antibodies specific for N-end rule substrates or their activated fragments.

15 Measurement of the degree of modification of the protein can be achieved in a binding assay format, preferably an ubiquitin specific format, available to a skilled artisan. Examples of techniques that can be used for detecting or measuring the degree of protein modification include mass spectrometry, chromatography, electrophoresis, agglutination, western blot, specific binding assays, immunoassay, immunofluorescence and immunochromatographic assays detected via surface plasmon resonance, radioactivity measurement, fluorescence, fluorescence polarization, phosphorescence, luminescence, chemiluminescence and electrochemiluminescence (ECL). The preferred method of the present invention is the specific binding assay with electrochemiluminescence detection system, preferably using the SectorHTS Reader and Multi-Spot Multi-Array plates (MSD).

The immunoassay or specific binding assay according to the preferred embodiments of the invention can involve a number of formats available in the art. The

5 antibodies and/or specific binding partners can be labeled with a detectable label or
immobilized on a surface. The term “antibody” includes intact antibody molecules
(including hybrid antibodies assembled by *in vitro* re-association of antibody subunits),
antibody fragments and recombinant protein constructs comprising an antigen binding
domain of an antibody (as described, e.g., in Porter and Weir, 1966, *J.Cell Physiol.*, 67
10 (Suppl 1); 51-64 and Hochman, Inbar and Givol, 1973, *Biochemistry* 12: 1130; hereby
incorporated by reference). The term also includes intact antibody molecules, antibody
fragments and antibody constructs that have been genetically altered or chemically
modified, e.g., by the introduction of a label.

Preferably, the detection is performed by contacting an assay composition with
15 one or more detection molecules capable of specifically binding with the marker(s) of
interest. More preferably, the assay uses a sandwich or competitive binding assays
format. Examples of sandwich immunoassays performed on test strips are described by
U.S. Patent No. 4,168,146 to Grubb et al. and U.S. Patent No. 4,366,241 to Tom et al.
Examples of competitive immunoassay devices suitable for use with the present
20 invention include those disclosed by U.S. Patent No. 4,235,601 to Deutsch et al., U.S.
Patent No. 4,442,204 to Liotta, and U.S. Patent No. 5,208,535 to Buechler et al. Most
preferably, at least one of the binding reagents employed in such an assay is immobilized
on a solid phase support, preferably on a surface of a tubes, multi-well plate, particle (e.g.
magnetizable particles), filter, porous membrane, electrode, slide and/or chip.

25 Examples of suitable experimental designs to measure ubiquitylation of a given
protein and/or variant thereof schematically are depicted in Figure 1.

5 In one preferred embodiment of the invention depicted in Figure 1(A), a labeled ubiquitin, preferably labeled with a detectable label, more preferably labeled with a radioactivity, fluorescence, fluorescence polarization, phosphorescence, luminescence, chemiluminescence or electrochemiluminescence label, more preferably labeled with ECL label, most preferably MSD TAGTM, is combined with the protein, a ubiquitylation
10 system and, optionally, additional unlabeled ubiquitin so that the labeled ubiquitin is incorporated into poly-Ub chains linked to the protein. The ubiquitylated products are captured on a surface, preferably on a surface of a tube, multi-well plate, particle (e.g. magnetizable particle), filter, porous membrane, electrode, slide and/or chip, most preferably a surface of a SectorHTS plate, via binding interaction of a labeled protein and
15 a binding partner immobilized on a surface. The extent of ubiquitylation is determined by measuring the amount of the ubiquitylated product, e.g., by measuring the amount of the detectable label on the surface. As shown, in the figure, the protein is labeled with a binding reagent (biotin) that binds to the immobilized binding partner (streptavidin, or alternatively avidin). Alternatively, the binding reagent is omitted and a binding partner
20 (e.g., an antibody) is employed that binds directly to the protein of interest. In another alternative embodiment, the protein of interest is directly immobilized on the surface (e.g., by passive adsorption or through covalent bonds).

 A variant of the embodiment depicted in Figure 1 (A) and described above is shown in Figure 1 (B). This embodiment differs in that the ubiquitylated product is
25 detected using a labeled ubiquitin specific binding reagent (preferably an antibody), preferably labeled with a detectable label, more preferably labeled with a radioactivity, fluorescence, fluorescence polarization, phosphorescence, luminescence,

5 chemiluminescence or electrochemiluminescence label, more preferably labeled with ECL label, most preferably an MSD TAG™ labeled antibody. The ubiquitin specific binding reagent is, preferably, selective for poly-ubiquitin chains or ubiquitin-protein conjugates relative to free monomeric ubiquitin.

In yet another preferred embodiment of the invention depicted in Figure 1 (C) a
10 target protein, preferably labeled with a detectable label, is combined with ubiquitin and an ubiquitylation system so that the target protein is ubiquitylated. The ubiquitylated product is captured on a surface, preferably on a surface of or a multiwell plate or an electrode, most preferably on a surface of a Sector HTS™ plate, via an ubiquitin-specific binding reagent (preferably, an antibody). The ubiquitin specific binding reagent is,
15 preferably, selective for poly-ubiquitin chains or ubiquitin-protein conjugates relative to free monomeric ubiquitin. The ubiquitylation product is detected using the detectable label as a reporter so as to measure the extent of ubiquitylation.

The target protein is labeled with a binding reagent (shown as biotin) that binds to a labeled binding partner (shown as streptavidin) having a second detectable label,
20 preferably a radioactivity, fluorescence, fluorescence polarization, phosphorescence, luminescence, chemiluminescence or electrochemiluminescence label, more preferably an ECL label, most preferably MSD TAG™. The binding partner is combined with the ubiquitylation product and the second label is measured to determine the extent of ubiquitylation. In a variation of this embodiment, the target protein is unlabeled and the
25 labeled binding partner (preferably, an antibody) is specific for the target protein. In another variation of the embodiment, the binding partner is omitted and the target protein is directly labeled with a radioactivity, fluorescence, fluorescence polarization,

5 phosphorescence, luminescence, chemiluminescence or electrochemiluminescence label, more preferably labeled with ECL label, most preferably MSD TAGTM.

According to a preferred embodiment of the invention the ubiquitylation assays are then repeated in the presence of inhibitors of the N-end rule pathway, preferably in the presence of Arg- β -Ala and/or Trp-Ala dipeptide inhibitors of the N-end rule pathway.

10 Comparison of the extent of the ubiquitylation of a target protein in the presence or absence of the inhibitors allows for the classification of ubiquitylation substrates as N-end rule substrates or, more specifically, as Type I, Type II or Type III substrates.

The invention further provides for measuring the time course of a ubiquitylation reaction both in the presence and absence of dipeptide inhibitors, preferably by binding
15 assay and/or by denaturing gel electrophoresis radiography detection system. The time course identifies Type I vs. II vs. III substrates and identifies substrates which undergo rapid or slow ubiquitylation. The bands on the denaturing gel electrophoresis radiograph for samples collected at different times during an ubiquitylation reaction allow the measurement of, e.g., (1) the time course of the production and degradation of a full
20 length protein; (2) the time course of the accumulation of N-terminal fragments cleaved by specific proteases, the accumulation of which has no dependence on the presence or absence of dipeptide inhibitors; (3) the size of such N-terminal fragments; (4) the time course for the accumulation of C-terminal fragments having exposed N-degrons, the accumulation of which are dependent on the presence or absence of the N-end rule
25 pathway inhibitors; (5) the size of such C-terminal fragments and (6) the time course for the accumulation of multi-site proteolysis products for some protein substrates.

5 One preferred embodiment of the invention is directed to identification of a
protease cleavage site that is cleaved to form a fragment having a destabilizing N-
terminal residue which is processed via the N-end rule pathway. A protease cleavage site
is identified by (i) forming a mixture of an N-end rule ubiquitylation substrate and a
protease necessary to activate a substrate, preferably the mixture contains one or more E3
10 ligase inhibitors and/or one or more proteasome inhibitors and (ii) identifying a protease
cleavage site. In yet another embodiment, the mixture is enriched and/or treated with
deubiquitylating enzymes prior to analysis. The method may also include repeating the
experiment in the presence and absence of proteasome inhibitors. Comparison of the
peptides formed under the two conditions allows for the identification of those fragments
15 that possess an N-degron (i.e., the fragments that are only observed in the presence of the
inhibitor).

The protease may be supplied, e.g., (i) in purified form; (ii) by *in vitro* protein
expression system, preferably cell lysate, most preferably in reticulocyte lysate, or (iii) by
transformed host-cells or cell-free systems under conditions such that the encoded
20 sequence is expressed and the protein is produced.

According to one preferred embodiment the cleavage products are analyzed to
determine the sizes of proteolytic fragments and thereby to determine the location of the
cleavage site by techniques available to a skilled artisan. The examples of such
techniques include but are not limited to gel electrophoresis using radiographic or
25 colorimetric detection systems, chromatography (e.g. size exclusion chromatography),
centrifugation, mass spectroscopy (preferably on fragment isolated and purified,
preferably isolated and/or purified by chromatographic techniques, such as size

5 exclusion, affinity or ion exchange chromatography) and/or filtration. These techniques may be further supplemented by peptide sequencing techniques, e.g., sequencing techniques based on chemical or enzymatic degradation or sequencing techniques based on mass spectrometric analysis. Preferably the location of the cleavage site is refined based on the knowledge of the destabilizing residues, sequence and fragment size
10 information, preferably computationally.

Yet in another preferred embodiment labeled amino acids, preferably radiolabeled amino acids, are incorporated into N- and C- terminal fragments of the protein and the fragments are subjected to sequencing to identify a protease cleavage site.

Yet in another embodiment of the present invention, the protease cleavage site is
15 identified via mutagenesis, preferably scanning mutagenesis. Mutations are introduced into protein sequence, preferably in a vicinity of a putative cleavage site, more preferably at the cleavage site, preferably a putative site identified by the above described techniques. Mutations that deactivate N-end rule ubiquitylation pathway identify the cleavage site.

20 One embodiment of the invention is a method for identifying proteases which expose a destabilizing N-terminal amino acid residue in a target protein (preferably, a protein selected from the list of proteins in Table 1). Proteases are identified by either (i) measuring a binding of a putative protease to an N-end rule substrate or an activated fragment thereof, preferably to an N-end rule substrate protein having a mutation at or in
25 close proximity to a cleavage site which prevents proteolysis, more preferably to an N-end rule substrate having a transition-state analog in place of a cleavable bond, most preferably selected from proteins listed in Table 1, and/or variants thereof; or (ii) by

5 measuring proteolytic cleavage of an N-end rule substrate, most preferably selected from proteins listed in Table 1, and/or variants thereof.

Therefore, one embodiment of the present invention involves screening putative proteases, preferably protease libraries, by (i) contacting a putative protease with an N-end rule substrate, preferably protein selected from a group listed in Table 1 and/or
10 variant thereof and (ii) identifying proteases which bind to an N-end rule substrate.

Another embodiment of the present invention involves screening putative proteases, preferably protease libraries, for proteases which bind to an activated fragment of an N-end rule substrate, preferably a protein selected from a group listed in Table 1 and/or variants thereof. The techniques available to a skilled artisan include, but are not limited
15 to affinity chromatography, preferably an affinity chromatography resin having immobilized one or more N-end rule substrates, activated fragments and/or variants thereof, preferably where N-end rule substrates have a mutation at or in close proximity to a cleavage site which prevents proteolysis, more preferably an N-end rule substrate having a transition-state analog in place of a cleavable bond, most preferably selected
20 from proteins listed in Table 1, and/or variants thereof.

Yet in another embodiment of the invention N-end rule substrates or activated fragments thereof, preferably to an N-end rule substrate protein having a mutation at or in close proximity to a cleavage site which prevents proteolysis, more preferably to an N-end rule substrate having a transition-state analog in place of a cleavable bond, most
25 preferably selected from proteins listed in Table 1, and/or variants thereof are immobilized on supports known in the art of solid phase binding assays and include surfaces of tubes, multi-well plates, particles (e.g., magnetizable particles), filters, porous

5 membranes, electrodes, slides and chips. One especially preferred solid phase support is
a carbon electrode surface, more preferably a surface of a carbon ink electrode, most
preferably a surface of a plasma-treated patterned carbon ink electrode. Putative
proteases are linked to a detectable label, selected from a radioisotope, a fluorescent
(including fluorescence polarization), phosphorescent, luminescent, chemiluminescent
10 and/or electrochemiluminescent compound, an enzyme, or an enzyme co-factor as a label
moiety, including binding species recognized by preferably labeled binding partner (e.g.,
biotin, streptavidin, epitope tags, His6, fluorescein, hapten, immunogen, GST and/or
GFP), preferably an ECL label. Yet in another embodiment the binding is measured in
the ELISA format available to a skilled artisan. Another suitable binding assay format is
15 described in a European Patent Application EP 1182458A1.

Yet another embodiment of the present invention involves screening putative
proteases, preferably protease libraries, for proteases which cleave an N-terminal
fragment of an N-end rule substrate, preferably a protein selected from a group listed in
Table 1 and/or variants thereof, preferably having blocked (or internal) N-degron, to
20 expose destabilizing N-terminal residue. The proteases are identified in a protease
activity assay that comprises combining the putative protease with the substrate and
measuring the extent of cleavage of the substrate, e.g., by measuring the loss of substrate
or production of cleavage products. The method may use, e.g., one of the many formats
for measuring protease activity that are available to a skilled artisan.

25 In one specific embodiment, the present invention involves screening protease
libraries for proteases which expose N-degron thereby activating N-end rule
ubiquitylation. The method comprises combining a putative protease with a substrate

5 having an internal N-degron and measuring the production of cleavage products having an exposed N-degron. Preferably, the assay further comprises contacting the products with an N-end rule ubiquitylation system and, optionally, a proteosome system and measuring the production of ubiquitylated or proteosome-degraded peptide fragments, e.g., by the detection techniques described above. The ubiquitylation and/or proteosome
10 systems may be provided by a eukaryotic cell lysate, preferably a reticulocyte lysate, preferably a rabbit reticulocyte lysate. Optionally, the protein is prepared in such a lysate to provide a composition comprising the protein, ubiquitylation system and proteosome system. Proteosome inhibitors may be added to allow measurement of ubiquitylated fragments without interference from proteosome degradation of the fragments.

15 The invention further provides a method of identifying E3 ligases by screening libraries of proteins for proteins that promote the ubiquitylation of an ubiquitylation substrate. The ubiquitylation activity can be measured, e.g., by any of the techniques described above.

In one embodiment, the method comprises (i) contacting putative E3 ligases,
20 preferably libraries of putative E3 ligases with N-end rule substrates (preferably, a protein listed in Table 1 or a fragment or variant thereof) and a ubiquitylation system and (ii) identifying E3 ligases, preferably E3 ligases with N-end rule activity. The activity measurement is done as described above, where the ubiquitylation of an N-end rule substrate protein and/or variant thereof, preferably a protein selected from a group listed
25 in Table 1 and/or variants thereof, is detected. Preferably, the ubiquitylation system is deficient in E3 activity, e.g., activity of endogenous E3 ligases is detectably lower than the activity of a putative ligase, preferably by at least two-fold, more preferably by at

5 least five-fold, more preferably by at least 10-fold. Most preferably the N-end rule degradation system lacks endogenous E3 ligases. In one embodiment, the activity of endogenous E3 ligases is inhibited by an inhibitor specific for the endogenous E3 ligase.

 In one preferred embodiment, the E3 ligases are identified by their ability to bind N-end rule substrates, or activated fragments of N-end rule substrates (preferably, a
10 protein listed in Table 1 or a fragment or variant thereof). The method comprises combining a putative E3 ligase with a substrate and measuring the formation of binding complexes comprising the substrate and the putative E3 ligase. The formation of the binding complex may be measured by binding assay methods known to one skilled in the art. In one example, the substrate is immobilized on a solid supports known in the art of
15 solid phase binding assays and include surfaces of tubes, multi-well plates, particles (e.g., magnetizable particles), filters, porous membranes, electrodes, slides and chips, more preferably on a surface of a well in a multiwell plate, or on a chromatographic resin. One especially preferred solid phase support is a carbon electrode surface, more preferably a surface of a carbon ink electrode, most preferably a surface of a plasma-treated patterned
20 carbon ink electrode.

 In one specific embodiment of the invention, the retardation in the retention time of a putative E3 ligase by a chromatographic support having immobilized substrates of N-end rule ubiquitylation, or activated fragments thereof, preferably selected from a group of proteins listed in Table 1, identifies E3 ligases.

25 In yet another embodiment, the method further comprises contacting the ubiquitylation reaction products with a proteosome system and monitoring the degradation of the N-end substrate and/or variant thereof. For example in one specific

5 embodiment of the invention a disappearance of a substrate labeled with a detectable label, preferably an ECL label is detected.

Another aspect of the invention relates to improved methods and systems for selecting or identifying active compounds, preferably biologically active compounds *in vitro*, *ex vivo*, *in vivo* and/or *in silico*, and, optionally, incorporating such compounds into
10 suitable carrier compositions in appropriate dosages to form pharmaceutical compositions.

According to one embodiment of the present invention, active compounds are identified by screening one or more candidate compounds by a screening assay comprising (i) combining a candidate compound, an N-end rule substrate (preferably a
15 protein listed in Table 1 or an activated fragment thereof), an N-end rule ubiquitylation system and, optionally, a protease that exposes a hidden N-degron in said substrate; (ii) measuring the production of ubiquitylated products and (iii) identifying compounds that modulate the rate of ubiquitylation. The method may further comprise comparing the measured production to that measured in the absence of the candidate compound to
20 determine if the candidate compound modulates the rate of ubiquitylation.

Preferably, the method further comprises testing said one or more active compounds for bioavailability, toxicity and/or biological activity *in vivo*. Preferably, the method further comprises synthesizing analogues of said one or more active compounds and further screening analogues for activity, bioavailability, biological activity and/or
25 toxicity.

In one embodiment, the active compounds are modulators, preferably inhibitors or promoters of the ubiquitin ligation, preferably inhibitors or promoters of the ubiquitin

5 ligation to an exposed destabilizing N-terminal residue. Preferably the active compounds are inhibitors or promoters of E3 ligase, preferably Type I/II/III-specific N-end rule ubiquitylation inhibitors or promoters. Yet in another embodiment the active compounds identified according to the methods of this invention are inhibitors or promoters of E1 ligase or E2 ligase.

10 Yet in another embodiment the active compounds identified according to the methods of this invention are protease activity modulators, preferably inhibitors or promoters of a proteolytic cleavage necessary to expose N-degron.

Another preferred embodiment of the present invention is a method for determining whether the active compound is a specific modulator of a protease, or a
15 ligase system. While the active compounds which modulate the activity of a ligase system will not inhibit or promote production and/or detection of activated fragments of substrate proteins having exposed N-degron, the modulators specific for a protease will inhibit or promote production and/or detection of activated fragments of substrate proteins. Therefore, according to one embodiment of the present invention, the method
20 further comprises measuring the accumulation of non-ubiquitylated activated fragments to determine if the active compound is an inhibitor of protease activity or ubiquitylation activity. By way of example, these fragments will appear as bands in a gel electrophoresis analysis of the assay products if the ubiquitylation activity is diminished or absent but the bands will be greatly diminished or absent in the presence of
25 ubiquitylation activity.

According to a particularly preferred embodiment, the method further comprises formulating the one or more compounds into drugs for administering to humans and/or

5 animals. To prepare a pharmaceutical formulation, a suitable carrier is added to one or more active compounds identified according to the method of the instant invention. A skilled artisan will find FDA approved additives in a National Formulary compiled by the U.S. Pharmacopeia, or may develop his own.

Preferably, the formulating comprises determining the suitable amount of the one
10 or more active compounds in the drug and mixing the suitable amount with one or more excipients and/or carriers. Preferably, the excepiant comprises sugar and/or starch.

In one specific embodiment of the instant invention the active compounds are biological, naturally occurring moieties such as natural biosynthesis products, small molecules, saccharides, lipids, nucleic acids, peptides and proteins extracted from
15 microorganisms, fungi, or plant or animal life forms, or produced by modern chemical and biological techniques. According to the instant invention the naturally occurring moieties include antibodies and antibody fragments, including naturally occurring antibodies and/or antibody fragments and the ones produced by recombinant, phage and other immunological means either *in vivo*, *ex vivo* or *in vitro*. Yet according to another
20 embodiment the active compounds are selected from synthetic compound libraries, including commercially available compound libraries and/or selected from the compound library expansion by means of modern combinatorial chemistry.

One embodiment of the invention relates to a method for selecting or identifying biologically active compounds from a library of compounds, said method comprising
25 screening said library of compounds for biological or biochemical activity, wherein said screening includes assaying the library of compounds for the biological or biochemical activity.

5 The invention includes the use of the methods of the invention to screen for new drugs, preferably, by high-throughput screening (HTS), preferably involving screening of greater than 50, more preferably 100, more preferably 500, even more preferably 1,000, and most preferably 5,000. According to a particularly preferred embodiment, the screening involves greater than 10,000, greater than 50,000, greater than 100,000, greater
10 than 500,000 and/or greater than 1,000,000 compounds. In one specific embodiment of the present invention the active compounds are selected from the libraries of FDA approved drugs, such as FDA2000.

Such screening and/or drug discovery methods include those set forth in U.S. Patent No. 5,565,325 to Blake; U.S. Patent No. 5,593,853 to Chen et al.; U.S. Patent No.
15 5,721,135 to Thatrup et al., U.S. Patent No. 5,985,585 to Daggett et al.; U.S. Patent No. 5,684,711 to Agrafiotis et al.; U.S. Patent No. 5,639,603 to Dower et al.; U.S. Patent No. 5,569,588 to Ashby et al.; U.S. Patent No. 5,541,061; U.S. Patent No. 5,574,656; and U.S. Patent No. 5,783,431 to Peterson et al.

According to another embodiment, the invention further comprises identifying
20 adverse effects associated with the drug and storing information relating to the adverse effects in a database. See, U. S. Patent No. 6,219,674 by Classen, hereby incorporated by reference.

The invention also includes bivalent inhibitors of E3 ubiquitin ligases, comprising a first moiety that binds the Type I site and a second moiety that binds the Type II site of
25 an N-end rule E3 ligase, preferably UBR1. The two moieties are covalently linked through one or more covalent bonds. Preferably, the two moieties are covalently linked through one or more linking moieties providing, e.g., a bivalent inhibitor with structure

5 M1-L-M2 where M1 is the first moiety, M2 is the second moiety and L is a linking moiety.

Suitable first and second moieties are, preferably, selected from moieties known to act as inhibitors of Type I or Type II N-end rule ubiquitylation and are, most preferably, Type I or Type II destabilizing amino acids. Preferably, the bivalent
10 inhibitors have molecular weights of less than 2000, more preferably less than 1200 and most preferably less than 950 Daltons. Surprisingly, we have found that such small molecules comprising Type I and Type II inhibitors not only inhibit both Type I and Type II activities but also show better inhibition than a mixture of the corresponding monovalent inhibitors. Although applicants do not wish to be bound by theoretical
15 explanations of the effect, such effect may be attributed to a stronger binding of a bivalent inhibitor to the E3 ligase through cooperative binding of the two moieties to the Type I and Type II sites.

In one embodiment of the invention, the bivalent inhibitors of the present invention are linear molecules. The Type I and Type II inhibiting moieties are,
20 preferably, separated by less than 90 bonds, more preferably by less than 50 bonds, more preferably by less than 36 bonds, even more preferably by less than 30 bonds.

In an especially preferred embodiment, the bivalent inhibitors are peptides, preferably of less than 30 amino acid residues, more preferably of less than 25 amino acid residues, more preferably of less than 20 amino acid residues, more preferably of less
25 than 12 residues.

Peptide-based inhibitors preferably comprise two N-terminal amino acids so as to provide Type I and Type II destabilizing residues. Such peptides may be made, e.g., by

5 including in the peptide a branching monomer that has two amino groups. Linkage of the amino groups to the C-terminal residues of two peptide sequences (a first peptide sequence and a second peptide sequence) results in a peptide-based inhibitor having two N-termini. The two peptide sequences are chosen so that the N-terminal residue of the first peptide sequence is recognized by the Type I site and the N-terminal residue of the
10 second peptide sequence is recognized by the Type II site. Especially preferred branching monomers are lysine and arginine, most preferably lysine where one of the peptide sequences is linked to the lysine via the alpha amino group and the other is linked via the epsilon amino group. The synthesis of branched peptides is easily accomplished by solid phase peptide synthesis and is well within the purview of the skilled artisan.

15 One embodiment of inhibitors of the present invention are inhibitors of general chemical formula:



where X is an amino acid (inhibitor of Type I site) selected from the group of

Arg, Lys and His;

20 Y is an amino acid (inhibitor of Type II site) selected from the group of

Phe, Leu, Trp, Tyr, and Ile;

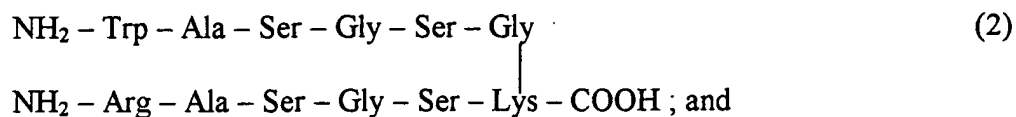
AA is any amino acid residue either naturally occurring, or synthetic;

B is a branching monomer, preferably Lys or Arg, most preferably Lys;

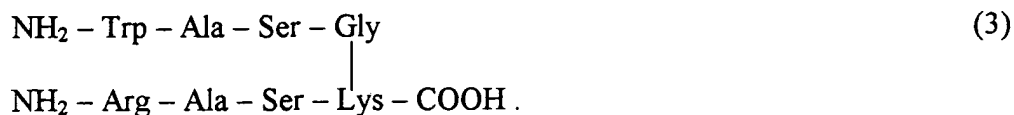
and

25 n and m are in between 0 and 30 and where n+m is less or equal to 30.

5 In some specific embodiments of the invention, the preferred inhibitors of the present invention are of chemical formulae:



10



In structures 2 and 3, the vertical bond coming from the lysine refers to a peptide
15 bond between the epsilon amino group of the lysine and the carboxyl group of the attached glycine.

In one preferred embodiment, the invention relates to modulating the abundance and/or activity of N-end rule ubiquitylation substrates *in vitro*, *ex vivo* or *in vivo*. The modulation is achieved by mutating a site of proteolytic cleavage necessary to expose N-
20 degon, preferably by mutating a key residue or by engineered a new cleavage site, thereby either retarding or accelerating degradation of an N-end rule substrate. In one preferred embodiment of the invention, the mutation is accelerating the substrate cleavage and/or the substrate clearance from the cell, thereby lowering the activity of the protein. Yet in another embodiment of the invention the mutation is inactivating the N-
25 end rule pathway, therefore creating an overpopulation of a substrate of interest. Such overpopulation is desirable for studying protein function, or analyzing a protein-dependent phenotype.

5 Yet in another preferred embodiment the abundance of a substrate is modulated by administering one or more active compounds identified according to the present invention.

Yet in another embodiment the invention is directed to a method for generating a phenotypic line (cell, or animal) by modulating the abundance of a protein via enriching or inactivating N-end rule pathway for a specific protein. Such mutation may be used in
10 a phenotypic background of an animal imitating a complex disease, such as for example modulating the abundance of protein tau in a background of a mouse overexpressing A β .

Another preferred embodiment the invention relates to a kit for producing and analysing polypeptides ubiquitylated via N-end rule pathway preferably polypeptides
15 selected from proteins listed in Table 1, or variants thereof. The kit components are conveniently designed to provide DNA fragments, for example in a form of cDNA clones, or cDNA clones incorporated into appropriate vectors, preferably cDNA clones which encode proteins listed in Table 1, or variants thereof and reagents necessary to produce desired protein sequences by *in vitro* coupled transcription translation as
20 described to the co-pending U.S. Patent Application 10/238,960, entitled "Methods, Reagents, Kits and Apparatus for Protein Function", incorporated herein by this reference. The kit may also include one or more assay components, such as (i) one or more binding reagents; (ii) a pH buffer; (iii) one or more blocking reagents; (iv) luminescent label; (v) luminescence co-reactant; (vi) preservatives; (vii) stabilizing
25 agents; (viii) enzymes; (ix) detergents; and (x) desiccants.

5 One specific embodiment relates to a kit which includes components required for active compound and/or pharmaceutical composition identification according to the methods of the instant invention.

 The invention is further directed to methods of treating disease by administering pharmaceutical compositions comprising modulators of N-end rule ubiquitylation, 10 preferably, modulators of the N-end rule ubiquitylation of a protein listed in Table 1. In certain embodiments of the invention, the modulator is a bivalent N-end rule inhibitor as described above.

 One embodiment of the invention is a method for treating ataxia, preferably ocular motor ataxia, or Freidreich ataxia, a disease which is characterized by loss of 15 aprataxin, a protein identified herein as an N-end rule ubiquitylation substrate. In one preferred embodiment of the invention, a therapeutic dose of an N-end rule ubiquitylation inhibitor, preferably a protease and/or ubiquitin ligase inhibitor, most preferably a protease inhibitor is administered into a patient in need thereof, thereby increasing the effective concentration of aprataxin. In another preferred embodiment, the disease is 20 treated using gene therapy by introducing a clone encoding aprataxin but having a mutated protease cleavage site, where cleavage at the site in the unmodified protein leads to exposure of an N-degron. The mutation is selected to i) prevent proteolytic cleavage at the site and/or ii) prevent the exposure, on cleavage at the proteolytic cleavage site, of an N-degron. Such mutation will inhibit N-end rule dependent degradation of aprataxin, 25 thereby increasing the lifetime of a protein, preferably reducing the necessary dose in gene therapy.

5 Another embodiment of the invention is a method for treating a neurodegenerative disorder selected from the group of neurodegenerative disorders generally characterized as tauopathies, preferably Alzheimer's disease, or Parkinson's disease, or Frontotemporal Dementia, which are characterized by neurofibrillary tangles formed predominantly from tau, a protein identified herein as an N-end rule ubiquitylation substrate. In one preferred embodiment of the invention, a therapeutic dose of an N-end rule ubiquitylation promoter is administered to a patient in need thereof, thereby accelerating the degradation of tau via the N-end rule dependent pathway. Yet another embodiment of the invention is a method of medical treatment by administering to a patient in need thereof a therapeutic dose of i) a protease which cleaves the tau protein and exposes an N-degron and/or ii) a ubiquitin ligase that ubiquitylates an activated fragment of tau having exposed an N-degron thereby accelerating tau degradation via the N-end rule dependent pathway.

 Another embodiment of the invention is a method for treating a neurodegenerative disorder selected from the group of neurodegenerative disorders generally characterized by a disruption in neurotransmission via disruption in membrane trafficking or other mechanisms, for example Alzheimer's disease and Griscelli syndrome, which are linked to an aberrant performance of SLP, a protein identified herein as an N-end rule ubiquitylation substrate. In one preferred embodiment of the invention, a therapeutic dose of an N-end rule ubiquitylation modulator is administered to a patient in need thereof. The modulator is chosen to decelerate (e.g., by inhibiting the N-end rule E3 or a protease or other enzyme responsible for exposing a hidden N-degron) or accelerate (e.g., by enhancing the rate of an N-end rule E3) the effective degradation of

5 a SLP via N-end rule dependent pathway. Deceleration is desirable, e.g., in the treatment of Alzheimer's diseases and acceleration is desirable, e.g., in the treatment of Griscelli syndrome. Yet another embodiment of the invention is a method of medical treatment by administering to a patient i) a therapeutic dose of a protease which cleaves the SLP protein and exposes an N-degron and/or ii) a ubiquitin ligase that ubiquitylates an
10 activated fragment of a SLP having an exposed N-degron thereby accelerating a SLP degradation via the N-end rule dependent pathway. Yet another embodiment of the invention is a method of medical treatment by administering to a patient a therapeutic dose of i) an inhibitor of a protease which cleaves the SLP protein and exposes an N-degron and/or ii) an inhibitor of a ubiquitin ligase that ubiquitylates an activated fragment
15 of a SLP having an exposed N-degron thereby decelerating a SLP degradation via the N-end rule dependent pathway.

Another embodiment of the invention is a method for treating a cancer that is characterized by the activation of a telomerase which can be inhibited by PinX1, a protein identified herein as an N-end rule substrate. In one preferred embodiment of the
20 invention, a therapeutic dose of an N-end rule ubiquitylation inhibitor is administered to a patient, thereby decelerating the effective degradation of PinX1 via N-end rule dependent pathway. Yet another embodiment of the invention is a method of medical treatment by administering to a patient a therapeutic dose of i) an inhibitor of a protease which cleaves the PinX1 protein and exposes an N-degron and/or ii) an inhibitor of a ubiquitin ligase
25 that ubiquitylates an activated fragment of PinX1 having an exposed N-degron thereby decelerating PinX1 degradation via the N-end rule dependent pathway and thereby inhibiting telomerase activity.

5 Another embodiment of the invention is a method for treating human disorders
selected from the group of human neoplastic disease, cerebral autosomal dominant
arteriopathy with subcortical infarcts and leucoencepholopathy, Alagille syndrome,
Epstein-Barr virus-induced immortalization (the process associated with human
malignancies such as Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease
10 and lymphoproliferative disease in immunosuppressed patients) which are linked to an
aberrant performance of CIR, a protein identified herein as an N-end rule ubiquitylation
substrate. In one preferred embodiment of the invention, a therapeutic dose of an N-end
rule ubiquitylation modulator is administered to a patient, thereby decelerating or
accelerating the degradation of a CIR via the N-end rule dependent pathway. Yet another
15 embodiment of the invention is a method of medical treatment by administering to a
patient i) a therapeutic dose of a protease which cleaves the CIR protein and exposes an
N-degron and/or ii) a ubiquitin ligase that ubiquitylates an activated fragment of a CIR
having an exposed N-degron thereby accelerating CIR degradation via the N-end rule
dependent pathway. Yet another embodiment of the invention is to method of medical
20 treatment by administering to a patient a therapeutic dose of i) an inhibitor of a protease
which cleaves the CIR protein and exposes an N-degron and/or ii) an inhibitor of a
ubiquitin ligase that ubiquitylates an activated fragment of a CIR having an exposed N-
degron thereby decelerating CIR degradation via the N-end rule dependent pathway.

 Another embodiment of the invention is a method for treating diseases and/or
25 disorders generally characterized by a disruption in protein processing and clearance
which involves a putative E3 ligase Cullin-3, a protein identified herein as an N-end rule
ubiquitylation substrate. In one preferred embodiment of the invention, a therapeutic

5 dose of an N-end rule ubiquitylation modulator is administered to a patient, thereby
decelerating (e.g., in case of overexpression of cyclin D1, a cullin-3 substrate, which has
been implicated in a variety of tumors such as breast cancers, gastrointestinal tumors and
lymphomas) the degradation of a Cullin-3 via the N-end rule dependent pathway and
improving target substrate clearance or accelerating (e.g., in case of katanin, a cullin-3
10 substrate, which has been implicated in a suppression of microtubule instability) the
degradation of a Cullin-3 via N-end rule dependent pathway thereby reducing the
clearance of a target protein. Yet another embodiment of the invention is a method of
medical treatment by administering to a patient i) a therapeutic dose of a protease which
cleaves the cullin-3 protein and exposes an N-degron and/or ii) a ubiquitin ligase that
15 ubiquitylates an activated fragment of a cullin-3 having an exposed N-degron thereby
accelerating cullin-3 degradation via the N-end rule dependent pathway. Yet another
embodiment of the invention is a method of medical treatment by administering to a
patient a therapeutic dose of i) an inhibitor of a protease which cleaves cullin-3 protein
and exposes an N-degron and/or ii) an inhibitor of a ubiquitin ligase that ubiquitylates an
20 activated fragment of cullin-3 having an exposed N-degron thereby decelerating cullin-3
degradation via the N-end rule dependent pathway.

One embodiment of the invention is a method for improving astrocyte
differentiation in response to CNS injury, which is achieved by modulating the
concentration of HMGN3, a protein identified herein as an N-end rule ubiquitylation
25 substrate. In one preferred embodiment of the invention, a therapeutic dose of an N-end
rule ubiquitylation inhibitor, preferably a protease and/or ubiquitin ligase inhibitor, most
preferably a protease inhibitor is administered to a patient, thereby increasing the

5 effective concentration of HMGN3. In another preferred embodiment, the disease is treated using gene therapy by introducing a clone encoding HMGN3 but having a mutated protease cleavage site, where cleavage at the site in the unmodified protein leads to exposure of an N-degron. The mutation is selected to i) prevent proteolytic cleavage at the site and/or ii) prevent the exposure, on cleavage at the proteolytic cleavage site, of an
10 N-degron. Such mutations will inhibit the N-end rule dependent degradation of HMGN3, thereby increasing the lifetime of a protein, preferably reducing the necessary dose in gene therapy. Yet another embodiment of the invention is a method of medical treatment by administering to a patient i) a therapeutic dose of an inhibitor of a protease which cleaves HMGN3 protein and exposes an N-degron and/or ii) an inhibitor of a ubiquitin
15 ligase that ubiquitylates an activated fragment of HMGN3 having an exposed N-degron thereby decelerating HMGN3 degradation via N-end rule dependent pathway.

Another embodiment of the invention is a method for treating certain lymphomas, which are characterized by aberrant lymphocyte apoptosis regulation by HSPC144, a protein identified herein as an N-end rule ubiquitylation substrate. In one preferred
20 embodiment of the invention, a therapeutic dose of an N-end rule ubiquitylation promoter is administered to a patient, thereby accelerating the degradation of HSPC144 via the N-end rule dependent pathway. Yet another embodiment of the invention is a method of medical treatment by administering to a patient i) a therapeutic dose of a protease which cleaves the HSPC144 protein and exposes an N-degron and/or ii) a ubiquitin ligase that
25 ubiquitylates an activated fragment of HSPC144 having an exposed N-degron thereby accelerating HSPC144 degradation via the N-end rule dependent pathway.

5 One embodiment of the invention is a method for for treating certain types of
cancers, which are characterized by downregulation of the expression of cdc6, a protein
identified herein as an N-end rule ubiquitylation substrate. In one preferred embodiment
of the invention, a therapeutic dose of an N-end rule ubiquitylation inhibitor, preferably a
protease and/or ubiquitin ligase inhibitor, most preferably a protease inhibitor is
10 administered to a patient, thereby increasing the effective concentration of cdc6. In
another preferred embodiment, the disease is treated using gene therapy by introducing a
clone encoding cdc6 but having a mutated protease cleavage site, where cleavage at the
site in the unmodified protein leads to exposure of an N-degron. The mutation is selected
to i) prevent proteolytic cleavage at the site and/or ii) prevent the exposure, on cleavage
15 at the proteolytic cleavage site, of an N-degron. Such mutations will inhibit N-end rule
dependent degradation of cdc6, thereby increasing the lifetime of a protein, preferably
reducing the necessary dose in gene therapy. Yet another embodiment of the invention is
a method of medical treatment by administering to a patient a therapeutic dose of i) an
inhibitor of a protease which cleaves cdc6 protein and exposes an N-degron and/or ii) an
20 inhibitor of a ubiquitin ligase that ubiquitylates an activated fragment of Cdc6 having an
exposed N-degron thereby decelerating Cdc6 degradation via the N-end rule dependent
pathway.

Another embodiment of the invention is a method for treating certain diseases,
which are characterized by aberrant cell differentiation regulation by HMGN2, a protein
25 identified herein as an N-end rule ubiquitylation substrate. In one preferred embodiment
of the invention, a therapeutic dose of an N-end rule ubiquitylation promoter is
administered to a patient, thereby accelerating the degradation of HMGN2 via the N-end

5 rule dependent pathway. Yet another embodiment of the invention is a method of medical treatment by administering to a patient i) a therapeutic dose of a protease which cleaves the HMGN2 protein and exposes an N-degron and/or ii) a ubiquitin ligase that ubiquitylatea an activated fragment of HMGN2 having an exposed N-degron thereby accelerating HHMGN2 degradation via the N-end rule dependent pathway.

10 A person of ordinary skill in the art will readily recognize the possible modification to the methods of treating diseases described above.

7. EXAMPLES

The following examples are illustrative of some of the methods falling within the
15 scope of the present invention. They are, of course, not to be considered in any way limitative of the invention. Numerous changes and modifications can be made with respect to the invention by one of ordinary skill in the art without undue experimentation.

Materials and Methods

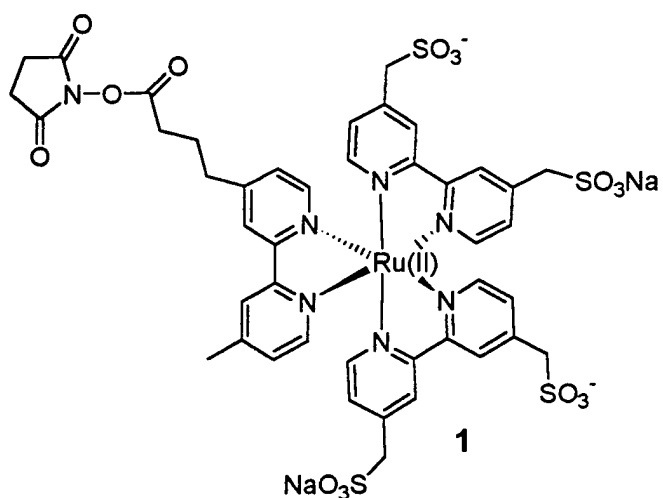
20 **Preparation of plasmid DNA:**

Studies on N-end rule substrates were conducted using proteins produced using the following IMAGE Consortium clones: IMAGE ID # 3994375 (apratxin), # 3887570 (synaptotagmin-like protein), # 4448167 (microtubule-associated protein tau), # 3447081 or #3455121 (both HMGN2), # 3890883 or #4749335 (both HMGN3), # 3867414 (cell
25 cycle controller Cdc6), # 4426807 (cullin 3), #3910222 (CBF1 interacting protein (CIR)), #3907737 (HSPC144) and # 3911679 (PIN2 interacting protein 1 (PINX1)). Bacteria

5 containing clones were grown in 25 ml LB with 250 $\mu\text{g/ml}$ Amp and DNA was isolated using QIAprep Spin Midiprep Kits (Qiagen). DNA yield was 100-150 μg .

Streptavidin Labeled with Electrochemiluminescent Labels:

The label Compound 1 pictured below (Sulfo-TAGTM NHS Ester, Meso Scale Discovery, a division of Meso Scale Diagnostics, LLC, Gaithersburg, MD) was used to
10 label streptavidin for electrochemiluminescence measurements.



Labeling of streptavidin was carried out by adding the Sulfo-TAG NHS Ester to a solution of the streptavidin molecules in phosphate buffered saline, pH 8.0. The labeled protein was purified from unbound label by size exclusion chromatography using
15 Sephadex G50 (Pharmacia Biosciences). The average number of labels per protein molecule (typically around 11) was calculated from the concentration of labels (calculated from the extinction coefficient of Sulfo-TAG label at 455 nm, $\epsilon_{455} \sim 15,400 \text{ M}^{-1}\text{cm}^{-1}$) and the concentration of streptavidin (determined using the BCA Protein Assay, Pierce Chemicals).

5 **Multi-Well Plates for Electrochemiluminescence Measurements:**

Electrochemiluminescence measurements were carried out using specially designed multi-well plates having integrated carbon ink electrodes for carrying out electrochemiluminescence measurements (Multi-Array™ or Multi-Spot™ plates, Meso Scale Discovery, a division of Meso Scale Diagnostics, LLC, Gaithersburg, MD). A dielectric layer patterned over the working electrode in each well exposed four regions or “spots” on the working electrode. The exposed working electrode in two of the spots was coated with FK2 (Affiniti Research Products) a mouse monoclonal antibody that is specific for poly-ubiquitylated proteins. Another spot was coated with BSA and used as a negative control. FK2 was diluted to 0.1-0.5 mg/ml with PBS, pH7.4, 0.015% Triton-X100, microdispensed (250 nl per spot) onto two spots of each well of a 4-Spot Multi-Spot plate and air-dried for 5 hours. Then 200 µl of a blocking solution (PBS, pH7.2, 5% BSA, 0.1% 2-chloroacetamide) was introduced into each well and blocking was performed overnight at 4°C. The plates were washed three times with PBS prior to use.

Electrochemiluminescence Measurement Instrument:

20 Electrochemiluminescence was induced and measured in the Multi-Spot plates using a Sector HTS™ reader (Meso Scale Discovery, a division of Meso Scale Diagnostics, LLC, Gaithersburg, MD).

Electrochemiluminescence Assay for Substrates of N-end Rule-Dependent Ubiquitylation:

25 Protein was produced from the plasmid DNA in a transcription-translation reaction mixture containing rabbit reticulocyte lysate and SP6 RNA polymerase (TNT SP6 Quick Mix, Promega) and that was further supplemented with 20 µM methionine, 8-

5 30 µg/ml plasmid DNA, 20 µg/ml biotinylated Lys-tRNA (transcend tRNA, Promega),
and 50 µM of the proteasome inhibitor MG-132 (Calbiochem) in a total volume of 12.5
µl. Ubiquitylation of newly synthesized proteins occurred in the same reaction mixtures
and was driven by the ubiquitylation system present in reticulocyte lysates. An ECL-
based ubiquitylation assay (described below) was used to determine if the newly
10 synthesized protein was ubiquitylated. To determine if the protein produced from each
clone was ubiquitylated through the N-end rule pathway, the ECL ubiquitylation assay
was repeated in the presence of 1 mM Arg-β-Ala (an inhibitor of ubiquitylation of Type I
N-end rule substrates), 1 mM Trp-Ala (an inhibitor of ubiquitylation of Type I N-end rule
substrates), 1 mM of each of the two dipeptide inhibitors, or, in some experiments, 1 mM
15 of bivalent inhibitor **3**, an inhibitor comprising peptide sequences having both N-terminal
Arg-Ala and Trp-Ala moieties. These reactions were carried out in the presence of 0.15
nM bestatin, an aminopeptidase inhibitor that was added to prevent degradation of the
dipeptide inhibitors.

The reactions were allowed to proceed for a desired period of time at 30°C. In
20 some experiments, several reaction time points were analyzed. Then 1 µl of each
reaction mixture was mixed with 50 µl binding buffer (20mM Tris-HCl, 150 mM NaCl,
pH7.4, 0.1%BSA, 0.2% Tween-20, 20 mM EDTA, and a cocktail of protease inhibitors
(Roche Applied Sciences)) containing 1µg/ml sulfotag-labeled streptavidin in the well of
the FK2-coated Multi-Spot plates. The plate was incubated on a tabletop shaker for 1
25 hour. During this time, newly synthesized proteins comprising both a biotin label and
poly-ubiquitin bind to both immobilized FK2 and Sulfo-TAG labeled streptavidin
resulting in the accumulation of Sulfo-TAG labels on the FK2 “spot”. Thereafter the

5 plate was washed three times with 20 mM Tris-HCl, 0.004% Triton X-100 followed by addition of 150 μ l of a buffered solution containing tripropylamine (MSD Read Buffer, Meso-Scale Discovery, Gaithersburg MD) into each well. ECL signals from labels bound to the electrode surfaces were measured using a Sector HTS instrument.

Analysis of N-end Rule-Dependent Degradation of Proteins by Denaturing Gel:

10 Proteins were synthesized as described in the previous section except that biotinylated Lys-tRNA was substituted with [14 C]-lysine, and MG-132 was omitted. The reactions were incubated at 30°C for indicated times. They were stopped by transferring of aliquots of reactions into SDS-sample buffer. The samples were heated for 5 min at 95°C and fractionated by SDS-PAGE on 4-20% gradient polyacrylamide gels. Gels were
15 soaked in AmplifyTM fluorographic reagent (Amersham Pharmacia Biotech), dried and exposed to X-ray films.

EXAMPLE 1: RGS4 a known N-end rule ubiquitylation substrate is confirmed as an N-end rule substrate

20 RGS4 was produced and characterized as an N-end rule substrate as described in the Materials and Methods section. RGS4 is a known Type 1 N-end rule ubiquitylation substrate (Davydov and Varshavsky, 2000, *J. Biol. Chem.*, 275: 22931).

Figure 2(A) shows the time course of the accumulation of ubiquitylated RGS4. Figure 2(B) shows the % of RGS4 ubiquitylation in the presence of specific N-end rule
25 inhibitors compared to the reaction in the absence of the inhibitors. Figures 2(A) and (B) show that the mixture of Arg- β -Ala and Trp-Ala dipeptides and Arg- β -Ala alone (Type I inhibitor) inhibit RGS4 ubiquitylation to a similar extent, while Trp-Ala alone (Type II

5 inhibitor) has little to no effect. These results identify RGS4 as a Type 1 substrate for an N-end rule ubiquitylation pathway.

The autoradiogram pictured in Figure 2(C) indicates that N-end rule ubiquitylation of RGS4 most likely proceeds after a proteolytic cleavage event exposes an N-degron. Figure 2(C) shows the time course of RGS4 ubiquitin/proteosome
10 degradation via N-end rule pathway in the presence or absence of a Arg- β -Ala or Trp-Ala dipeptide inhibitors of E3 ubiquitin ligase. The C-terminal fragment of a RGS4 having the N-terminal Met removed shows a time dependent accumulation exclusively in the presence of Arg- β -Ala inhibitor, confirming it as a Type I N-end rule ubiquitylation substrate. In the absence of the inhibitors the fragment is presumably degraded via the N-
15 end rule pathway.

EXAMPLE 2: Microtubule-Associated Protein Tau is an N-End Rule Ubiquitylation Substrate

Microtubule-Associated Protein Tau (MAPT, tau) was produced using IMAGE
20 clone # 4448167 and analyzed for its ability to act as an N-end rule substrate by ECL assay and by gel electrophoresis as described in the Materials and Methods.

Figure 3(A) shows the time course of the accumulation of ubiquitylated tau. Figure 3(B) shows the % of tau ubiquitylation in the presence of specific N-end rule inhibitors compared to the reaction in the absence of the inhibitors. Figures 3(A) and (B)
25 show that the mixture of Arg- β -Ala and Trp-Ala dipeptides and Arg- β -Ala alone (Type I inhibitor) inhibit tau ubiquitylation to a similar extent, while Trp-Ala alone (Type II

5 inhibitor) has little to no effect. These results identify tau as a Type 1 substrate for an N-end rule ubiquitylation pathway.

The autoradiograms pictured in Figures 3(C) and 3(D) indicate that N-end rule ubiquitylation of tau most likely proceeds after a proteolytic cleavage event exposes an N-degron. Figure 3(C) shows the time course of tau ubiquitin/proteasome degradation
10 via N-end rule pathway in the presence or absence of a mixture of Arg- β -Ala and Trp-Ala dipeptide inhibitors of E3 ubiquitin ligase. Figure 3(D) is an overexposed version of Figure 3(C). The proteolytic degradation of tau appears to be a complex process involving multiple cleavage events, as apparent from the complex ladder formation seen on the gel. However, the time dependent accumulation of ~ 15-20 kDa activated
15 fragment of tau exclusively in the presence of the inhibitors (Fig. 3(D) pointer) identifies tau as an N-end rule ubiquitylation substrate. In the absence of the inhibitors, this fragment is presumably degraded via N-end rule pathway.

EXAMPLE 3: Aprataxin is an N-End Rule Ubiquitylation Substrate

20 Aprataxin was produced using IMAGE clone # 3994375 and analyzed for its ability to act as an N-end rule substrate by ECL assay and by gel electrophoresis as described in the Materials and Methods.

Figure 4(A) shows the time course of the accumulation of ubiquitylated aprataxin. Figure 4(B) shows the % of aprataxin ubiquitylation in the presence of specific N-end
25 rule inhibitors compared to the reaction in the absence of the inhibitors. Figures 4(A) and (B) show that the mixture of Arg- β -Ala and Trp-Ala dipeptides and Arg- β -Ala alone (Type I inhibitor) inhibit aprataxin ubiquitylation to a similar extent, while Trp-Ala alone

5 (Type II inhibitor) has little to no effect. These results identify aprataxin as a Type 1 substrate for an N-end rule ubiquitylation pathway.

Figure 4(A) also shows that, at equivalent concentrations, bivalent peptide inhibitor **2** provides better inhibition of aprataxin ubiquitylation than the mixture of Arg- β -Ala and Trp-Ala dipeptides and Arg- β -Ala alone. Although applicants do not wish to
10 be bound by theoretical explanations of the effect, such effect may be attributed to a stronger binding of a bivalent inhibitor to E3 ligase due to cooperative binding at two different sites.

The autoradiogram pictured in Figure 4(C) indicates that N-end rule ubiquitylation of aprataxin most likely proceeds after a proteolytic cleavage event
15 exposes an N-degron. Figure 4(C) shows the time course of aprataxin ubiquitin/proteosome degradation via N-end rule pathway in the presence or absence of a mixture of Arg- β -Ala and Trp-Ala dipeptide inhibitors of E3 ubiquitin ligase. Band 1 (aprataxin) shows a time dependent retardation in the degradation of an aprataxin in the presence of the inhibitors. The retardation is seen predominantly at later time points.
20 Bands 2 (~20-21.5 kDa C-terminal fragment of aprataxin) and 3 (~ 16.5-18kDa N-terminal fragment of aprataxin) are derived from the starting molecule in band 1 via a single proteolytic cleavage. Band 2 (C-terminal fragment of aprataxin) shows a time dependent accumulation exclusively in the presence of inhibitors, confirming it as an N-end rule ubiquitylation substrate. In the absence of the inhibitors, the fragment is
25 presumably degraded via the N-end rule pathway. Finally, band 3 corresponds to N-terminal fragment of aprataxin – a product of a proteolytic cleavage necessary to expose N-degron.

5 The molecular weights of the bands place the protease cleavage site between residues 150-160 of aprataxin. The exact site is identified by scanning mutagenesis starting with residues Glu, Gln, Cys, Arg, Lys, His specific to Type I N-end rule substrate within this segment, or by mass-spectroscopy.

10 **EXAMPLE 4: Synaptotagmin-Like Protein 2 is an N-End Rule Ubiquitylation Substrate**

Synaptotagmin-like protein 2 was produced using IMAGE clone # 3887550 and analyzed for its ability to act as an N-end rule substrate by ECL assay and by gel electrophoresis as described in the Materials and Methods.

15 Figure 5(A) shows the time course of the accumulation of ubiquitylated synaptotagmin-like protein 2. Figure 5(B) shows the % of synaptotagmin-like protein 2 ubiquitylation in the presence of specific N-end rule inhibitors compared to the reaction in the absence of the inhibitors. Figures 5(A) and (B) show that the mixture of Arg- β -Ala and Trp-Ala dipeptides and Trp-Ala alone (Type II inhibitor) inhibit synaptotagmin-like
20 protein 2 ubiquitylation to a similar extent, while Arg- β -Ala alone (Type I inhibitor) has little to no effect. These results identify synaptotagmin-like protein 2 as a Type II substrate for an N-end rule ubiquitylation pathway.

Figure 5(A) also shows that bivalent peptide inhibitor 2 provides better inhibition of synaptotagmin-like protein 2 ubiquitylation than the mixture of Arg- β -Ala and Trp-
25 Ala dipeptides and Trp-Ala alone.

The autoradiogram pictured in Figure 5(C) indicate that N-end rule ubiquitylation of synaptotagmin-like protein 2 most likely proceeds after a proteolytic cleavage event

5 exposes an N-degron. Figure 5(C) show the time course of an synaptotagmin-like protein
2 ubiquitin/proteosome degradation via N-end rule pathway in the presence or absence of
a mixture of Arg- β -Ala and Trp-Ala dipeptide inhibitors of E3 ubiquitin ligase. Band 1
(synaptotagmin-like protein 2) shows a time dependent retardation in the degradation of a
synaptotagmin-like protein 2 in the presence of the inhibitors. Band 2 (C-terminal
10 fragment of synaptotagmin-like protein 2) is derived from a starting molecule in band 1
via a single proteolytic cleavage that removes a small N-terminal fragment of no more
than 50 amino acid residues. Band 2 (C-terminal fragment of synaptotagmin-like protein
2) shows the time dependent accumulation exclusively in the presence of inhibitors,
which identifies it as an N-end rule ubiquitylation substrate. In the absence of the
15 inhibitors, this fragment is presumably degraded via N-end rule pathway. Finally, the
lack of observed N-terminal fragment of synaptotagmin-like protein 2 – a product of a
proteolytic cleavage necessary to expose N-degron may result from a cleavage site
located very close to the N-terminus of synaptotagmin-like protein 2, most likely within
the first 50 amino acids.

20 The data identified the cleavage site to be located within the first 50 amino acid
residues of synaptotagmin-like protein 2. The site location is refined by scanning
mutagenesis starting with residues Leu, Ile, Tyr, Phe, Trp specific for Type II substrate,
within the first 50 amino acids, or by mass-spectroscopy.

The cleavage site is also identified when Synaptotagmin-like protein 2 is
25 produced using IMAGE clone # 3887550 having an engineered affinity purification tag
which allows capture of an N-terminal fragment followed by sequencing.

5 **EXAMPLE 5: HMGN2 (HMG17) is an N-End Rule Ubiquitylation Substrate**

High Mobility Group Chromosomal Protein 17 (now renamed HMGN2) was produced using IMAGE clone # 3455121 and analyzed for its ability to act as an N-end rule substrate by ECL assay as described in Materials and Methods. Alternatively IMAGE clone # 3447081 may be used.

10 Figure 6(A) shows the time course of the accumulation of ubiquitylated HGMN2. Figure 6(B) shows the % of HMGN2 ubiquitylation in the presence of specific N-end rule inhibitors compared to the reaction in the absence of the inhibitors. The figures 6(A) and (B) show that the mixture of Arg- β -Ala and Trp-Ala dipeptides and Arg- β -Ala alone (Type I inhibitor) inhibit HMGN2 ubiquitylation to a similar extent, while Trp-Ala alone
15 (Type II inhibitor) has little to no effect. These results identify HMGN2 as a Type 1 substrate for an N-end rule ubiquitylation pathway.

EXAMPLE 6: Cell Cycle Controller Cdc6 is an N-End Rule Ubiquitylation Substrate

20 Cell Cycle Controller cdc6 was produced using IMAGE clone # 3867414 and analyzed for its ability to act as an N-end rule substrate by ECL assay and by gel electrophoresis as described in the Materials and Methods.

Figure 7(A) shows the time course of the accumulation of ubiquitylated cdc6. Figure 7(B) shows the % of cdc6 ubiquitylation in the presence of specific N-end rule
25 inhibitors compared to the reaction in the absence of the inhibitors. Figures 7(A) and (B) show that the mixture of Arg- β -Ala and Trp-Ala dipeptides and Arg- β -Ala alone (Type I inhibitor) inhibit cdc6 ubiquitylation to a similar extent, while Trp-Ala alone (Type II

5 inhibitor) has smaller effect. These results identify cdc6 as a Type 1 substrate for an N-end rule ubiquitylation pathway.

The autoradiogram pictured in Figure 7(C) indicates that N-end rule ubiquitylation of cdc6 most likely proceeds after a proteolytic cleavage event exposes an N-degron. Figure 7(C) show the time course of a Cell Cycle Controller cdc6
10 ubiquitin/proteosome degradation via N-end rule pathway in the presence or absence of a mixture of Arg- β -Ala and Trp-Ala dipeptide inhibitors of E3 ubiquitin ligase. The proteolytic degradation of Cdc6 appears to be a complex process involving multiple cleavage events, as apparent from the complex ladder formation seen on the gel. The protein is clearly unstable as evidenced from a disappearance of a major band (Cdc6
15 ~55kDa) after 2 hours. However, the cdc6 band at about 55kDa shows slight time dependent retardation in its degradation in the presence of the inhibitors. The retardation is seen predominantly at medium (~2hrs) time points. Also bands at about ~ 40-45 kDa shows a time dependent accumulation exclusively in the presence of inhibitors, which confirms cdc6 as an N-end rule ubiquitylation substrate. In the absence of the inhibitors
20 the fragment is presumably degraded via the N-end rule pathway.

EXAMPLE 7: HSPC144 is an N-End Rule Ubiquitylation Substrate

HSPC144 was produced using IMAGE clone # 3907737 and analyzed for its ability to act as an N-end rule substrate by ECL assay as described in the Materials and
25 Methods.

Figure 8(A) shows the time course of the accumulation of ubiquitylated HSPC144. Figure 8(B) shows the % of HSPC144 ubiquitylation in the presence of

5 specific N-end rule inhibitors compared to the reaction in the absence of the inhibitors. Figures 8(A) and (B) show that the mixture of Arg- β -Ala and Trp-Ala dipeptides and Arg- β -Ala alone (Type I inhibitor) inhibit HSPC ubiquitylation to a similar extent, while Trp-Ala alone (Type II inhibitor) has no effect. These results identify HSPC144 as a Type 1 substrate for an N-end rule ubiquitylation pathway.

10

EXAMPLE 8: PIN2 Interacting Protein 1 (PinX1) is an N-End Rule Ubiquitylation Substrate

PinX1 was produced using IMAGE clone # 3911679 and analyzed for its ability to act as an N-end rule substrate by ECL assay as described in the Materials and
15 Methods.

Figure 9(A) shows the time course of the accumulation of ubiquitylated PinX1. Figure 9(B) shows the % of PinX1 ubiquitylation in the presence of specific N-end rule inhibitors compared to the reaction in the absence of the inhibitors. Figures 9(A) and (B) show that the mixture of Arg- β -Ala and Trp-Ala dipeptides and Arg- β -Ala alone (Type I
20 inhibitor) inhibit PinX1 ubiquitylation to a similar extent, while Trp-Ala alone (Type II inhibitor) has no effect. These results identify PinX1 as a Type I substrate for an N-end rule ubiquitylation pathway.

EXAMPLE 9: HMGN3 is an N-End Rule Ubiquitylation Substrate

25 HMGN3 was produced using IMAGE clones # 3890883 and # 4749335 and analyzed for its ability to act as an N-end rule substrate by ECL assay as described in Materials and Methods.

5 Figures 10 (A) and (C) show the time course of the accumulation of ubiquitylated
HMGN3 encoded by clones # 3890883 and # 4749335, respectively. Figures 10 (B) and
(D) (HMGN3 encoded by clones # 3890883 and # 4749335 respectively) show the % of
HMGN3 ubiquitylation in the presence of specific N-End Rule inhibitors compared to the
reaction in the absence of the inhibitors. Figure 10 shows that the mixture of Arg- β -Ala
10 and Trp-Ala dipeptides and Arg- β -Ala alone (Type I inhibitor) inhibit HMGN3
ubiquitylation to a greater extent, while Trp-Ala alone (Type II inhibitor) has little effect.
The effect is most pronounced for clone # 4749335 (Fig. 10 (C-D)). These results identify
HMGN3 as a Type I substrate for an N-end rule ubiquitylation pathway.

15 **EXAMPLE 10: CBF1-interacting Protein (CIR) is an N-End Rule Ubiquitylation
Substrate**

CIR was produced using IMAGE clone # 3910222 and analyzed for its ability to
act as an N-end rule substrate by ECL assay as described in the Materials and Methods.

Figure 11 (A) shows the time course of the accumulation of ubiquitylated CIR.
20 Figure 11 (B) shows the % of CIR ubiquitylation in the presence of a mixture of N-End
Rule inhibitors compared to the reaction in the absence of the inhibitors. The Figure 11
shows that CIR is a substrate for an N-end rule ubiquitylation pathway.